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PHOTORECEPTORS IN MYA ARENARIA, WITH SPECIAL REFERENCE TO THEIR DISTRIBUTION, STRUCTURE, AND FUNCTION[1]

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SEVEN TEXT FIGURES AND ONE PLATE (ELEVEN FIGURES)

AUTHOR'S ABSTRACT

Experimental studies on the responses to light in Mya arenaria L. indicate that photosensitive tissue is located somewhere near the inner surface of the siphon and that the siphon is sensitive throughout its entire length.

A histological study of the siphon shows cells of a special type in the photosensitive region, which are most abundant where the inner surface of the siphon is most sensitive. They are found throughout the length of the siphon, just beneath the inner epithelial layer, around both the incurrent and excurrent siphons. They receive nerve elements from branches of sixteen large nerves.

Each cell contains a characteristic inner structure, the optic organelle, composed of a rather large hyaline structure, the lens, which is surrounded by a network of nerve fibrillae, the retinella. Light rays, reflected from a flat mirror through the lens in these cells, are brought to a focus in the region of the retinella, irrespective of the direction of the rays.

The cells are similar in structure and function to visual cells in leeches and photoreceptors in the earthworm. Available data indicate that they function as photoreceptors and that the fibrillae of the retinella are direct receptors of light stimuli.

Pigment spots found in considerable number on the distal third of the siphon, and thought by some to be eyespots, are, owing to simulation of the background, probably protective in nature, rather than functional in photoreception.

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INTRODUCTION

Poli (1795) was probably the first to maintain that some of the lamellibranchiate molluscs possess eyes. He pointed out that Ostrea (Pecten) jacobaea and Spondylus gaederopus possess small bright structures distributed along the edge of the mantle in considerable numbers, and he asserted that these structures are eyes. Since then eyes have been found and studied in various bivalve molluscs by Krohn ('44), Siebold ('48), Hensen ('65), Gegenbaur ('78), Sharp ('83, '84), Carrière ('85, '89), Patten ('86), Thiele ('89, '90), Retzius ('92), Hesse ('00), Küpfer ('16), and others. In some forms, e.g., Pecten, Arca, Spondylus, Lima, and Pectunculus, the eyes are well developed. In others, e.g., Solen, Venus, Cardium, Mactra, and Mya, the presence of eyes is questionable, as indicated by the following.

Will ('44) states that in Cardium, at the tip of each of the thread-like tentacles, projecting from the distal end of the siphon, there is a sunken eye; but Carrière ('85) says that these structures are not eyes, and Leydig ('57) contends that he could not find any eyes in other similar molluscs. Ryder ('83), however, holds that in Mya arenaria there are, near the free end of the siphon, pigmented papillae which serve as eyes. He says: "do not the habits of Ostrea, . . . justify us in expecting to find rudimentary organs on the mantles and siphons of molluscs, answering the purpose of eyes, as appears to be the case in the instance of Solen?"

However this may be, it has long since been known that many molluscs in which no eyes are evident respond very definitely to light. These responses have been studied more extensively in Mya arenaria than in any other related form, especially by Wenrich ('16), Hecht ('19, '20), Piéron ('25, '26), and Folger ('27), but these investigators did not consider the sensory structures involved in the responses. The present investigation was undertaken to ascertain the location, the nature, and the function of these structures. The conclusions reached are based upon a study of the response to light under various conditions and upon a histological study of the siphons.

MATERIAL AND METHODS

This investigation was begun at the Marine Biological Laboratory at Woods Hole and continued at The Johns Hopkins University. The specimens used at Woods Hole were furnished by the supply department of the laboratory. Those used at The Johns Hopkins University were obtained from sand bars in that portion of the Severn River called Round Bay, about 6 miles up the river from Annapolis.[2] An abundance of specimens was collected here at different times and taken to the laboratory.

A dozen or more of the individuals from the first collection were put into a large aquarium containing sand, water, and plants from the same locality, after which the water was aerated. In this collection there was one specimen much smaller than the rest. This one, with two other small molluscs of a different species, brought into the laboratory at the same time, was put into a battery jar together with débris and water. The water was not aerated, and no particular attention was paid to it for some time. The specimens in the aquarium all died within three weeks, while at the end of six weeks the three animals in the battery jar were still alive, and appeared to be in good condition. Fewer animals, which had just been collected, were now put into the same large aquarium with a fresh supply of water, sand, and plants. These animals lived slightly longer than the first group, but not as long as desired. It occurred to me, then, that perhaps the aerated specimens were receiving too much oxygen. Other specimens were therefore procured and put into individual battery jars, each containing brackish water, sand, and water plants, but the water was not aerated.

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[2] The animals in this region have very brittle shells, probably owing to deficiency in calcium, so that in obtaining them special precautions must be taken to avoid crushing them.

TABLE 1

Length of life in Mya arenaria under laboratory conditions. Thirty-eight specimens used

	NUMBER OF ANIMALS THAT DIED DURING EACH OF THE FOLLOWING THIRTY-DAY PERIODS										
	1 to 30	31 to 60	61 to 90	91 to 120	121 to 150	151 to 180	181 to 210	211 to 240	241 to 270	271 to 300	Total number
Date collected Dec. 10					2		1		1		4
Date collected Feb. 25	4	2	1	3	1	3	1			1	16
Date collected Apr. 21	1	2	3		3	3	3	3			18
Total	5	4	4	3	6	6	5	3	1	1	38
Average length of life in days	19	43	65	117	134	159	187	220	241	294	125

Kellogg ('99) maintains that adult Mya die quickly in the laboratory. Some of the specimens in the battery jars, however, lived more than eight months. The approximate length of life of thirty-eight specimens, collected at three different times, is recorded in table 1. By referring to this table one will see that two animals remained alive under laboratory conditions for a period exceeding eight months, and that one of these lived over nine months. This individual, collected February 25th, had about three-fourths of the siphon removed by an operation September 25th. The animal recovered successfully from the effects of the operation, and lived until December 15th—a period of 294 days. The average length of life of all the specimens was 125 days; twenty-two lived longer than the average, while only sixteen did not live as long as the average, and the greatest mortality occurred during the first thirty-day period, in the group of animals gathered February 25th. This high mortality can be attributed to two factors: February 25th was a very cold day, and some of the animals were injured in digging them from the partly frozen sand bar. Then, too, large numbers of specimens of the crustacean Gammarus were brought into the laboratory and put into the jars with the molluscs. This crustacean is a scavenger, and it was thought that it would eat the dead and decaying plant and animal life which gathered in the aquaria. This proved to be true, but it also attacked the molluscs, and caused the death of several of them.

THE DISTRIBUTION OF PHOTOSENSITIVE TISSUE AS INDICATED BY RESPONSE TO LIGHT UNDER VARIOUS CONDITIONS

A. Normal animals

Nature of response. Mya arenaria responds both to an increase and to a decrease in luminous intensity. Wenrich ('16) maintains that a different set of muscles is involved in the reaction under the two conditions. He says:

> For decreases in intensity the movement was that of the muscles involved in closing the siphonal openings, while in responses to increases, the siphon tubes were withdrawn. In the latter case the withdrawal of the siphon tubes was sometimes accompanied by closure of the openings, especially if the reaction was a vigorous one, but as a general rule, when the movement of the siphon tube was not great, no closure of the openings resulted.

In the following observations responses to increase of intensity only are considered. These responses consisted in withdrawal of the siphon, and often closing of the siphonal openings, in accord with Wenrich's statement. But usually only the tip of the siphon contracted; the proximal two-thirds moved little or not at all, and after a response the animal usually soon recovered and extended the siphon to its former position. The period between illumination and the beginning of contraction is known as the reaction time.

Response to localized photic stimulation. A device for projecting a small beam of light was constructed in order to ascertain the location of photic sensitivity in the siphon and other parts of the animals. This was done by placing a Ford headlight bulb, connected to three dry cells, in a light-tight box attached to the tube of a microscope (Patten, '15). A wooden block with a small circular aperture at the center was placed in the tube of the microscope about 1 inch from the light. A similar wooden block was also placed near the lower end of the tube where the objective was attached. With this arrangement of light and screens, it was possible to obtain a minute beam of rather intense light.

In order to obtain illumination of approximately the same intensity and the same size of field in each test, a thin wire was attached rigidly to the objective of the microscope tube. This wire was made of such length that when the point was brought close to an object, the area illuminated was about 1 mm. in diameter. The wire was adjusted so that the tip coincided with the outer edge of the circular area illuminated.

For use in the experiments made to ascertain the relation between response and localized photic stimulation, a group of thirty-one animals of various sizes was selected; then each animal was put into a battery jar in the laboratory, containing just enough water from the stock aquarium to cover it. Five or six of these battery jars were now taken into the dark-room and placed on a table, at intervals of about a foot and a half, after which each one was covered with a light-tight box and left for from two and one-half to three hours for dark adaptation. Then, with the room in darkness except for just enough light, supplied by a ruby lamp,[3] to enable objects to be discernible, the light-tight cover was carefully removed from one of the jars; after which the device for projecting a small beam of light, described above, was adjusted for stimulation of the base of the siphon. Then the light in the apparatus was turned on, and at the same time a stop-watch was started. The watch was stopped at the instant the siphon began to contract in response to the stimulating light. The time was then recorded, after which the light-tight box was again placed over the jar. In this way each of the rest of the five or six animals in the dark-room was subjected to localized photic stimulation at the base of the siphon, then the process was repeated for each of the animals, with the stimulation located elsewhere.

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[3] It was found that the animals did not at any time respond to the illumination produced by the ruby lamp.

In this manner, the relation of response to localized photic stimulation was ascertained for the projecting foot; the edge of the mantle; the outer surface of the siphon at base, middle, and tip; and the inner surface of the incurrent and the excurrent siphons.[4] It required about ten minutes for one test of each animal in the group, so that a period of dark adaptation of ten minutes intervened between two successive stimulations of the same animal.

After the observations of the animals in this group were completed, another group of five or six individuals was taken into the dark-room and observations made on them in the same way. This procedure was continued until the thirty-one animals selected were tested.

No responses were observed when the edge of the mantle or the projecting foot was illuminated as described. The results obtained by illuminating other regions are presented in table 2. By referring to this table the following will be seen:

The entire siphon in Mya arenaria is sensitive to lateral photic stimulations, but the tip is most sensitive. Of the thirty-one specimens tested, twenty-four responded to stimulation at the tip, twelve to stimulation at the middle, and nine to stimulation at the base. The base of the siphon is, however, only slightly sensitive, for although nine individuals of

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[4] In the smallest animals the siphon was so small and so nearly transparent that it was impossible to see any specific difference in the localization of the stimulus dependent upon direction of illumination. Only results obtained for lateral illumination are therefore recorded for these.

TABLE 2

Relative photic sensitivity of different parts of the siphon in Mya arenaria; area illuminated, 1 mm. in diameter; intensity, 12.4 m.c.

DESIGNATION OF SPECIMEN	LENGTH OF SHELL IN CM.	LENGTH OF EXTENDED SIPHON IN CM.	REACTION TIME (IN SECONDS) WITH ILLUMINATION					
			Of outer surface at			Of inner surface of		
			Base of Siphon	Middle of siphon	Tip of siphon	Incurrent siphon	Excurrent siphon	Parts of both siphons
23	10.0	7.0	N	6.0	4.0	3.5	3.7	3.6
1	9.0	8.0	N	N	5.0	2.0	4.0	3.4
26	8.0	12.0	N	N	N	–	–	4.0
24	6.5	1.0	N	C	N	C	C	C
2	6.0	6.5	N	N	N	3.2	–	–
3	6.0	4.0	N	N	3.2	–	–	2.8
21	6.0	7.0	N	N	N	N	N	2.2
27	6.0	8.0	N	N	C	C	C	C
29	5.5	6.0	N	N	3.2	–	–	2.6
25	5.5	12.0	N	N	N	C	C	C
10	5.5	6.0	N	N	2.4	1.8	2.0	–
9	5.5	5.0	N	9.8	3.8	3.4	3.4	4.0
6	5.5	4.0	N	N	2.4	3.6	3.0	4.0
11	5.0	7.0	N	N	3.8	–	–	2.4
12	5.0	3.5	N	4.0	3.1	–	–	2.5
22	5.0	3.5	4.2	N	2.4	–	–	2.2
28	5.0	7.0	N	N	3.0	–	–	C
7	4.5	0.0	–	–	–	–	–	–
8	4.5	8.0	N	4.2	4.0	–	–	2.4
14	4.0	1.5	3.4	3.0	2.8	C	C	C
18	3.5	3.5	6.6	C	3.0	C	C	C
17	3.5	1.5	2.4	3.6	2.0	–	–	1.6
4	3.3	3.0	5.0	4.0	3.0	–	–	2.4
16	3.0	3.5	N	3.8	2.8	–	–	2.0
15	2.5	3.5	N	N	2.8	–	–	2.8
5	1.4	.75	N	1.6	1.4	–	–	1.6
30	1.1	.5	1.8	2.1	2.0	–	–	–
19	1.0	.5	1.6	1.5	1.5	–	–	–
20	1.0	.3	C	1.5	1.3	–	–	–
31	1.0	.4	1.4	C	1.2	–	–	–
13	.75	.3	1.4	C	1.4	–	–	–

N, no response; C, siphon closed; –, not stimulated.

the group responded, the reaction time varied greatly, and it was obtained only in the small animals, which are in general more sensitive than large ones. The response to stimulation is more rapid when the inner surface of the siphons is illuminated than when the outer surface is illuminated, the reaction time being shorter in all but three of the specimens tested, and in these three there was very little difference. The inner surfaces of the incurrent and the excurrent siphons are approximately equally sensitive. No response to localized photic stimulation is obtained when the siphons are closed.

The fact that the reaction time is shorter when the inner surface of the siphon is illuminated than when the outer is illuminated seems to indicate that the photosensitive tissue is located somewhere near the inner surface. Since, however, the results were obtained only in one intensity of illumination and with but a small area illuminated, they cannot be considered conclusive. Consequently, a similar study was made, extending over a wide range of luminous intensities, with larger areas illuminated.

Response to light of different intensities with large areas illuminated. All work on the reaction of Mya to light of different intensities was performed by means of apparatus arranged as illustrated in figure 1. In a dark-room a 1000-watt, concentrated filament, projection, Mazda lamp was mounted in a ventilator shaft (containing an opening 4 cm. square), in such a way as to produce a horizontal beam of light (fig. 1, A); and a 500-watt lamp was mounted in a light-tight box (also containing an opening 4 cm. square), in such a way as to produce a vertical beam of light (fig. 1, B). Various intensities of light were obtained by the use of a series of Wratten neutral-tint filters placed in the path of the rays of light. The different filters in this series transmitted, respectively, 0.01, 0.0476, 0.1, 1.0, and 10.3 per cent of the incident light. The voltage of the current used was frequently adjusted with a rheostat in the circuit, but in spite of this it varied considerably, resulting in a corresponding variation in the intensity of the light used. However, the maximum varia-

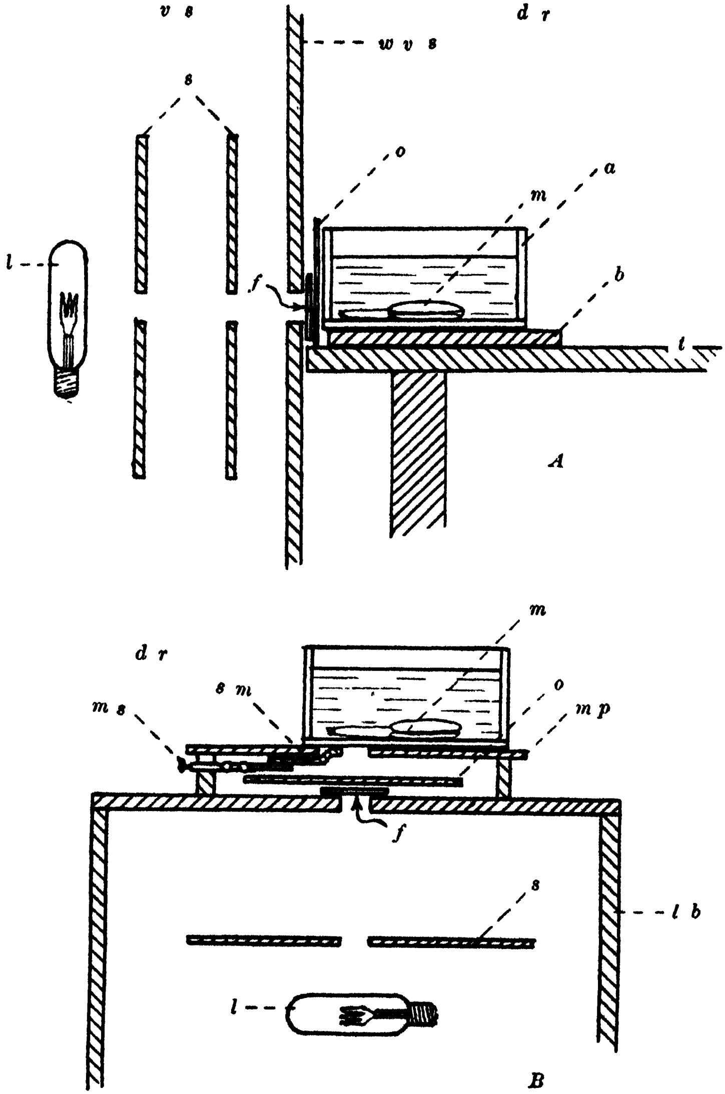

Figure 1

tion from that recorded in the tables never exceeded 12 per cent, and that obtained in only a few tests.

Each of six animals selected was taken from its battery jar in the laboratory and put into a small rectangular, plate-glass aquarium containing sufficient water from the battery jar to cover it. Then the animals were put upon a table in the dark-room, faintly illuminated with light from a ruby lamp, covered with light-tight boxes, as in the previous experiments, and left for two and one-half hours, i.e., until they were thoroughly dark adapted, after which the light-tight box was removed from one of the animals, and the aquarium containing it carefully transferred to a position before the opening in the wall of the ventilator shaft and so arranged that the distal end of the siphon was directed toward the lamp (fig. 1, A). The densest Wratten filter and an opaque screen were now placed between the source of light and the aquarium containing the animal. Then the light was turned on, and after it had attained its maximum intensity, the screen was removed and the reaction time ascertained as in the preceding tests. The light was then turned off, the animal returned to its place on the table and covered again with the light-tight box. This process was now repeated with each of the other five specimens on the table, after which it was repeated with all six specimens laterally illuminated (fig. 1, B). The Wratten filter used was now replaced by another, and the whole process repeated. This was continued until each of the thirty-one animals was alternately subjected to photic stimulation of the inner surface and the outer surface of the siphon in each intensity, and the reaction time for each animal was recorded.

Fig. 1 A. Apparatus used for illumination of the inner surface of the siphons of Mya arenaria and for lateral illumination of excurrent and incurrent siphons separately. B. Apparatus used for lateral illumination of different portions of the siphon. *a*, aquarium; *b*, block of wood; *dr*, dark-room; *lb*, light box; *m*, Mya; *mp*, metal plate with an opening for light to pass through; *ms*, mechanical stage; *o*, opaque screen; *s*, screens with openings for light to pass through; *sm*, sheet of metal attached to mechanical stage; *t*, table; *vs*, ventilator shaft; *wvs*, wall of ventilator shaft.

If an average is taken of the results obtained for all the individuals in each intensity and these averages are arranged as in table 3, the results show that reaction to illumination directed into the siphon is more rapid than the reaction to lateral illumination, and more rapid with the siphon open than with it closed. This supports the conclusion reached in the preceding section that the photosensitive tissue is located near the inner surface of the siphon, but it does not support the conclusion that Mya does not respond to photic stimulation when the siphons are closed.

When the reaction times are grouped in relation to the size of the animals and the average of each group is arranged as in table 4, the results again show that the reaction time is shorter for illumination directed into the siphons than for lateral illumination at the same intensity, thus again indicating that the photosensitive tissue is near the inner surface of the siphon. They also support the conclusion expressed in the section on localized photic stimulation, that small animals are more sensitive than large ones; and they show that there is a progressive increase in the reaction time as the animals increase in size.

TABLE 3

Relative effect of lateral illumination of siphons and illumination directed into the siphons; thirty-one animals used

INTENSITY IN M C.	REACTION TIME, IN SECONDS, WITH			
	Lateral illumination of siphons		Illumination directed into siphons	
	Open	Closed	Open	Closed
22567.8	1.69	2.01	1.50	1.81
2280.4	1.95	2.25	1.62	2.01
228.1	2.26	2.68	1.87	2.28
23.3	2.67	3.13	2.14	2.63
10.9	2.74	2.65	2.30	2.68
2.3	3.43	3.63	2.88	3.32

If the photosensitive tissue is located near the inner surface of the siphon as the results indicate, then one would expect about the same reaction time in large and small individuals if the inner surface of the siphon is equally illuminated. Since, however, the facts show that the smaller animals have a shorter reaction time than the larger ones, the possibility exists that the photosensitive tissue is located somewhere else than near the inner surface of the siphon.

The question then arises: Why, under the conditions which obtained in the preceding experiment, is the reaction time shorter in small specimens than in large ones, with the inner surface of the siphon illuminated? This may be due to the following: the photosensitive tissue may be so located that light must pass through a greater thickness of siphon wall in the large specimens to strike it; the photosensitive tissue illuminated in the various animals may be more sensitive in small specimens than in larger ones; or a greater quantity of photosensitive tissue may have been illuminated in the small individuals than in the large ones.

TABLE 4

Relation between size and sensitivity; with (L) lateral illumination and with (D) illumination directed into the siphons

Length of animals (cm.)		3.25	4.5	5.0–5.75	6.0–6.75	7.0–7.75	8.0–8.75
Number of animals used		1	2	11	10	4	3
INTENSITY IN M C		REACTION TIME IN SECONDS					
22567.8	L	1.0	1.8	1.52	1.77	1.9	2.2
	D	1.0	1.3	1.46	1.52	1.75	1.95
2280.4	L	1.4	1.7	1.89	2.11	2.15	2.5
	D	1.2	1.47	1.54	1.73	1.8	1.75
228.1	L	1.9	2.6	2.01	2.26	2.5	3.05
	D	1.5	1.7	1.63	2.0	2.0	2.2
23.3	L	2.2	2.73	2.58	2.37	3.9	3.55
	D	1.7	1.95	2.22	2.17	2.35	2.7
10.9	L	2.6	2.85	2.58	2.70	3.35	4.0
	D	2.1	2.1	2.26	2.42	2.25	2.7
2.3	L	2.9	3.1	3.63	3.56	3.3	4.3
	D	2.8	2.8	2.96	2.88	2.83	–

In subjecting animals to localized photic stimulations, an area having a diameter of 1 mm. in the small animals represents a relatively larger portion of the siphon than in the

the tip calls forth a response in approximately the same time as illumination of the middle of the siphon; and that illuminating a large area of the tip produces a more rapid response than when a small area is illuminated.

These facts again indicate that the photosensitive tissue is near the inner surface of the siphon, but that if it extends the entire length of the inner surface of the siphon, the wall of the siphon absorbs most of the rays of light in the larger animals; and that, if this is true, the responses to illumination at the middle and base of the siphon are probably due to the effect of light reflected into the open siphons, as indicated by the responses obtained with the water only illuminated.

TABLE 6

Relation between size of area illuminated and response. Fifteen animals used

SIZE OF ANIMAL (CM.)	DESIGNATION OF ANIMAL	REACTION TIME (SECONDS) TO LATERAL ILLUMINATION AT TIP OF SIPHON	
		Figures taken from individual readings used in preparing table 3; intensity 10.9 m.c. (4 cm. of siphon illuminated)	Figures taken from table 2, in an intensity of 12.4 m.c. (circular area 1 mm. in diameter illuminated)
5.5	6	2.4	2.4
5.5	9	2.45	3.8
5.5	10		2.4
5.5	29		3.2
5.5	51	2.3	
5.5	52	2.85	
5.25	50	2.3	
5.0	11		3.8
5.0	12		3.1
5.0	22		2.4
5.0	28		3.0
5.0	46	2.0	
5.0	70	2.9	
4.5	8	2.9	4.0
4.5	66	2.8	
Average		2.54	3.12

The fact that illuminating a large area laterally at the tip of the siphon calls forth a response in less time than illuminating a small area is supported by the results in table 6. The figures in this table were obtained from table 2, in which small circular areas 1 mm. in diameter were illuminated in an intensity of 12.4 m.c., and from the individual readings used in preparing table 3, in which the entire distal end of the siphon to a point about 4 cm. from the tip was illuminated in an intensity of about 10.9 m.c. The animals selected for comparison varied in length between 4.5 and 5.5 cm., inclusive. Only those readings were recorded in which responses to illumination were obtained with the siphons open. This table shows that the average reaction time with the smaller area exposed was 3.12 seconds, while with the larger area exposed in approximately the same luminous intensity it was only 2.54 seconds, thus indicating that the time required for response is dependent upon the quantity of photosensitive tissue illuminated.

The results presented in the preceding paragraphs show that the reaction time is dependent upon the intensity of illumination; the location of the photosensitive tissue; and the quantity of photosensitive tissue exposed to illumination; and that in the larger animals, when relatively equal portions of siphons are subjected to lateral illumination, there is an increase in the reaction time as the location of the stimulus proceeds from the tip toward the base.

The fact that an increase in the reaction time is obtained as the location of the stimulus proceeds from the tip toward the base of the siphon indicates either that the photosensitive tissue diminishes in quantity or quality toward the base of the siphon or that the siphon wall increases in opacity. In order, then, to ascertain which of these obtains, the response to light in specimens with various portions of the siphons removed was investigated.

B. Operated animals

Response to light with various portions of siphon removed. Animals that have been kept in aquaria in the laboratory under normal conditions usually have their siphons considerably extended. Such animals can be lifted and transferred to another container with but little contraction, but if the siphon is touched or merely jarred, it usually contracts suddenly. To cut off a definite portion of the siphon, it was therefore thought necessary to anaesthetize the animals.

Two per cent solutions of either cocaine or benzyl alcohol[5] injected into the siphon wall by means of a hypodermic needle produced local anaesthesia, but the portions of the siphons cut off were to be used for histological study later, and it was not known what effect the anaesthetic might have on the cell structure, and moreover, many animals died from the effects of the anaesthetic. An attempt was therefore made to operate on the siphons without the use of an anaesthetic.

Specimens with their siphons considerably extended were selected for operation. Each animal was carefully placed in a shallow pan, then the siphon was suddenly cut off with a sharp pair of scissors. These specimens withstood the effects of the operation very much better than those which were anaesthetized. Of twelve which were operated several times each, all recovered from the effects of the first, five from the effects of the second, three from the effects of the third, and two from the effects of the fourth operation. This operation, consequently, does not appear to be a great shock to the animal.

To ascertain the response to light with various portions of the siphon removed, twelve specimens were selected. Then the response to light with illumination directed into the siphon openings was ascertained for each specimen, after which a quarter of the length of the siphon was removed from each (fig. 4). All were now returned to the aquaria and left a day or two, after which they were again tested for responses to light. Then they were left a week or more, i.e., until they had completely recovered. Half of the siphon was now removed, after which the entire process was repeated as before. In the same way three-fourths of the siphon, and later as nearly the entire siphon as possible, were removed, the response to light being obtained after each operation.

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[5] Benzyl alcohol was first used as an anaesthetic by Macht, '18.

It soon became apparent that only a small number of the original animals selected would be able to survive four successive operations. Therefore, twelve more animals were selected, and in these the siphon was cut off as near the base as possible. After recovery, it was found that all of the animals had various sizes of short stumps of siphons remaining, one of which was about a quarter of the original length. The response to light was now obtained for this animal, after which as nearly the entire siphon as possible was removed; then the responses to light of all of the specimens were obtained. The results obtained are presented in table 7. These results show that the shortest reaction time in all but two intensities was obtained when approximately half of the siphon was removed, and in both of these it was shorter than under all other conditions, excepting when three-fourths of the siphon was removed. The table also shows that, with the siphons entirely removed or nearly so, the reaction time was considerably longer than it was with any other portion of the siphons removed. In the majority of tests under these conditions no response was observed at any luminous intensity, and in those in which responses were obtained it was invariably found that the siphon of the specimen tested was not entirely removed. As a matter of fact, it seemed evident that the longer the stump, the more likely the individual was to respond.

TABLE 7

Relation between the reaction time of all animals and the intensity of illumination; with siphons open; and with various lengths of siphon removed

QUANTITY OF SIPHON REMOVED	NUMBER OF ANIMALS USED	REACTION TIME, IN SECONDS, IN THE FOLLOWING INTENSITIES IN M C					
		19907.0	4649.7	501.8	47.4	23.5	5.0
None	12	1.78	2.01	2.17	2.55	2.61	3.23
One-fourth	12	1.65	1.91	2.24	2.81	3.06	3.57
One-half	5	1.54	1.68	1.92	2.34	2.38	3.32
Three-fourths	4	1.6	1.8	1.92	2.3	2.52	3.85
Entire	12	2.85	2.83	3.38	4.7	N	N

N, no response to stimulation.

In the preceding sections of this paper evidence was presented which shows that illumination of the inner surface of the siphon produces more rapid response than lateral illumination, thus indicating that the photosensitive tissue lies somewhere near the inner surface of the siphon, and that it extends throughout the length of the siphon. The results here presented support these conclusions, but, contrary to the conclusion reached in the preceding section that the tip of the siphon is most sensitive, they show that the middle portion is most sensitive. If this is true, there must either be more photosensitive tissue at the middle than elsewhere or the tissue that is there must be more sensitive to illumination than that found in other parts of the siphon.

Further evidence concerning the location and quantity of photosensitive tissue in the various parts of the siphon will be presented in the section on a histological study of the siphons.

Regeneration. In order to ascertain whether or not regeneration of the siphon takes place in Mya, a portion was removed in several specimens, after which they were put into the aquaria from which they had been taken. These animals were examined with a hand lens from time to time, a few of them for a period of several months, and they were occasionally tested for reaction to light.

The stumps healed completely within a few days. New tentacles formed around both the incurrent and excurrent siphon openings in about a month. The new tentacles developed directly from the new tissue formed in the healing of the wound. The regenerated tentacles were very small at first, but they grew, until in two months they were as long as the tentacles on normal specimens of the same size. Regeneration was, however, confined to reformation of new tentacles. There was no indication of increase in length of the siphon.

The results presented in table 8 are typical of those obtained after regeneration occurred. These results show that the reaction time after regeneration is practically the same as that immediately following the operation, i.e., that it does not increase while regeneration is taking place. This indicates that there is no regeneration of photosensitive tissue, although there is regeneration of new tentacles. This conclusion was confirmed by a histological study of regenerated tissues, for in no case was photosensitive tissue found in regenerated parts.

TABLE 8

Relation between sensitivity and regeneration in specimen 24 before removal of siphon, soon after its removal, and after regeneration; operation performed September 25th; animal died December 15th

INTENSITY IN M.C	REACTION TIME (SECONDS)			
	Before operation Sept 25	After operation Sept. 27	After regeneration	
			Nov 16	Dec. 10
53114.7	1.2	N	N	2.2
5457.2	1.4	2.8	N	N
505.4	1.6	N	N	N
51.1	1.9	N	N	N
24.5	2.1	N	N	N
5.2	2.3	N	N	N

N, no response obtained.

DISTRIBUTION AND STRUCTURE OF PHOTOSENSITIVE TISSUE AS INDICATED BY A STUDY OF THE STRUCTURE OF THE SIPHON

Distribution of nerves in the siphon

Information in the literature concerning the nerves in the siphon of Mya is very meager. By far the best description is given by Duvernoy ('54). In his drawing he represents two excurrent and two incurrent siphon nerves which are distinguished from each other, but he gives nothing concerning the finer details of these nerves and the endings of the fibers. Rawitz ('87) discusses the nervous system of Mya, but he figures only two nerves running to the siphon, and he gives no details concerning these. Essentially the same is shown in a drawing by W. R. Brooks, published in Packard's Zoölogy ('89).

The siphon nerves were studied both in gross dissection and in serial sections. For the dissection of the larger nerves, the animals were placed in a 4 per cent solution of ethyl alcohol. After a few hours, they were partially anaesthetized and the siphons were considerably extended and expanded; formalin was then added in small quantities until it reached a strength of about 10 per cent. In this solution they were kept until they were used in dissection. Before dissection they were washed in running water until nearly all the formalin was removed.

In dissecting the siphons they were slit lengthwise the entire length, along the midventral side of the incurrent siphon, and along the septum separating the incurrent and excurrent siphons; and then pinned down on a tray partly filled with wax, after which all the large nerves were dissected from the surrounding tissue.

For detailed dissection of smaller nerves, the method employed on earthworms by Hess ('25) was used. Small portions of the siphon were pinned open under water in Petri dishes partly filled with wax. The water was then drawn off and several drops of 2 per cent osmic acid placed on the region to be dissected. The dishes were covered to prevent the acid fumes from escaping, and left for about 10 minutes. At the end of this time, all the nerves on or near the surface were black, so that under a binocular microscope they could easily be dissected from the surrounding tissue. Since only the exposed portions of nerves are affected by the osmic-acid treatment, the process was repeated as often as necessary until dissection was complete.

Methylene blue was also used in dissection. Small pieces of tissue were placed in a Petri dish and covered with a methylene-blue solution (1 gram in 1000 cc. H_2O). After remaining in the stain for ten to fifteen minutes, the pieces were washed in water and then placed in a normal salt solution and dissected as described above. After a short time, however, the stain washed out, and no successful methods to make it permanent were found.

The methods used in the preparation of serial sections will be discussed presently.

The visceral ganglia in Mya arenaria lie side by side on the anteroventral side of the posterior adductor muscle, and are so closely associated that they appear as one ganglion (fig. 2).

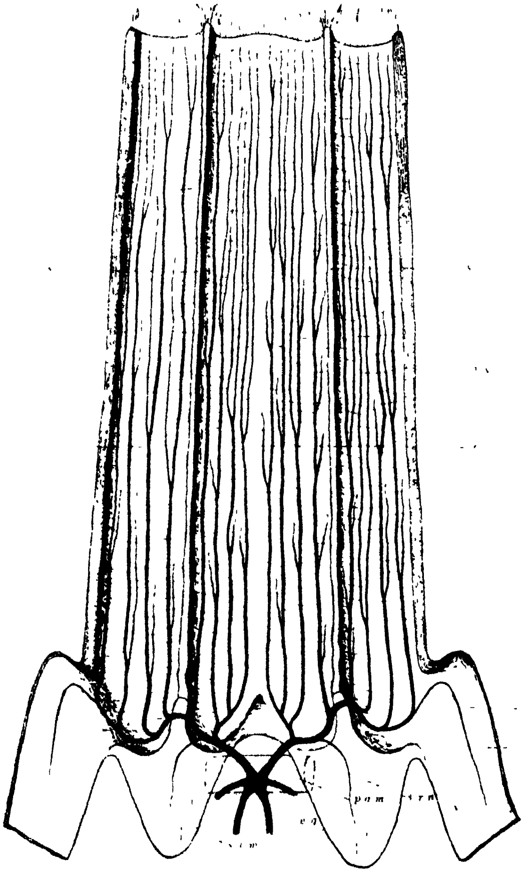


Fig. 2 Drawing showing the arrangement of the larger nerves in the siphon of Mya arenaria. The incurrent siphon and the septum separating the incurrent and excurrent siphons are slit lengthwise, and the siphon chambers are laid bare. *is*, incurrent siphon; *es*, excurrent siphon; *m*, mantle; *pam*, posterior adductor muscle; *pn*, pallial nerve; *s*, septum separating incurrent and excurrent siphons; *srm*, siphon retractor muscle; *srm*, siphon retractor nerve.

Each of the two ganglia sends a large nerve trunk posteriorly into the corresponding side of the animal. Each trunk soon divides, one part continuing posteriorly into the corresponding half of the excurrent siphon, while the other part continues as the pallial nerve, passing along between the siphon and the siphon retractor muscle, and bends anteriorly on the ventral side of the animal, innervating the corresponding half of the fused mantle. Soon after dividing, the pallial nerve gives off a small branch which passes anteriorly into the siphon retractor muscle. Five large branches are sent off posteriorly from the pallial nerve. One of these goes to the excurrent siphon near the wall separating the two siphons, and the other four to the incurrent siphon. That portion of the nerve trunk leaving the visceral ganglion, which upon dividing goes to the excurrent siphon, soon divides and sends off three branches into different parts of its half of the excurrent siphon. Thus each lateral half of each siphon is innervated by four large nerves which are connected directly with the central nervous system. These nerves, sixteen in number, extend the entire length of the siphon and send off branches which complete the innervation of the siphons.

A drawing of the cross-section of the siphon (fig. 3) shows that the main siphon nerves are embedded in the longitudinal muscles of the siphons and lie closer to the inside of the siphon than to the outside. There are no large nerves lying in the wall or septum separating the incurrent and excurrent siphons, but two large nerves on each side of the siphon, lying outside the extremities of this septum, send branches into it at intervals. All of the main nerves lie in a circle, at about the same distance, around the two siphon openings, a trifle more than a third of the distance from the inner wall of the siphon toward the outer wall.

Any part of the body of an animal which possesses as many nerves as the siphon of Mya must be capable of much nervous activity. When one remembers that these animals, normally buried in the sand, protrude only the tips of their siphons above the surface and that because of this habit they can keep in touch with their surroundings only by means of the siphons, then the utility of so great an innervation becomes apparent.

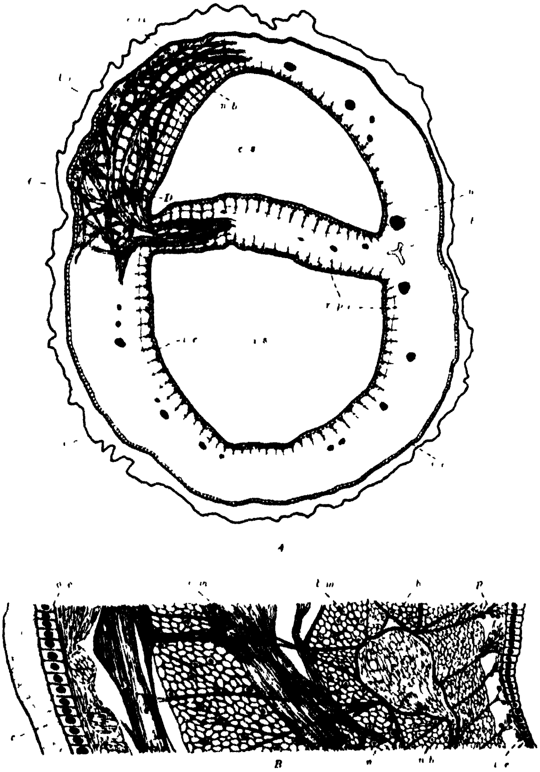


Fig. 3 A. Camera drawing of cross-section of siphon; gold-chloride technique. *b*, blood-vessel; *c*, cuticulum; *cm*, circular muscles; *es*, excurrent siphon; *ie*, inner epithelial cells; *is*, incurrent siphon; *lm*, longitudinal muscles; *n*, nerve trunks; *nb*, nerve branches; *oe*, outer epithelial cells; *p*, photoreceptors; *rpc*, region of photoreceptors. B. Greatly enlarged cross-section of a small portion of the wall of the siphon, through *C–D* (fig. 3, A), showing relation of photoreceptors and nerves. Labeling same as above.

Histological study of the siphons

Although previous workers, Will ('44), Ryder ('83), Sharp ('84), and Carrière ('85), reported the presence of eyes at the tip or the base of the tentacles of various molluscs closely related to Mya, Leydig ('57) claimed he could not find any eyes in these molluscs.

There is nothing in the literature on the structure of the photosensitive tissue in Mya arenaria or in any other closely related mollusc. Kellogg ('92) presents drawings of cross-sections through the body of Mya at various places, one through the siphon near the base, but he confines his drawings to gross structure.

For histological study, the best preparations were obtained by using the technique employed by Hess ('25) on earthworms. His method is a variation of Boule's modification of Cajal's silver-nitrate technique. The finer structure of the nerves in the photosensitive tissue was brought out more clearly by this method than by any other. For small nerves and nerve endings, the gold-chloride method as described in Guyer's Micrology was used. The results were satisfactory, but the gold chloride did not penetrate to the deeper tissues in which the principal large siphon nerves are located. Transparent preparations of tissue according to the method of Field ('22) proved very satisfactory in showing the structure of the photosensitive tissue and its connection with the central nervous system. Other material for histological study was fixed with Bouin's, Flemming's, and chrom-aceto-formol fixing fluids, and stained with Mallory's triple connective-tissue stain or Heidenhain's iron hematoxylin. The sections for histological study were cut uniformly 12 μ thick.

A study of the sections shows that the large nerves which extend the length of the siphon send a great number of branches toward the inner part of the siphon. By tracing these branches to their extremities, numerous pear-shaped cells were found, lying just beneath and sometimes in contact with the inner epithelial layer of the siphon. These cells are directly connected with the central nervous system and take on the characteristic silver-nitrate stain for nerve tissue. They are clearly different from any other cells found in the siphon.

The drawing (fig. 3) and the photomicrographs (figs. 8 and 9) show the location of these cells in cross-sections taken a short distance below the tip of the siphon and in a longitudinal section. They lie immediately beneath the inner epithelial layer of both siphons and normally extend entirely around the canals in both, but in fixing and staining, these canals assume a triangular outline in cross-sections, resulting in elimination of the cells at the apices of the angles. They seem to be more numerous in the septum separating the two siphons than elsewhere, although no actual counts were made.

The photomicrograph (fig. 10) shows a highly magnified portion of the section shown in figure 8, taken a short distance below the tip of the siphon. This is a typical section prepared with the silver-nitrate technique (Hess, '25). It shows the pear-shaped cells and their location in the siphons very clearly. These cells are entirely absent in the tentacles.

Distribution of the pear-shaped cells

In order to ascertain the distribution of the pear-shaped cells in the siphon, a histological study was made of ten adjoining serial sections, each 12 μ thick, taken from each of the regions designated *A*, *B*, *C*, *D*, *E*, and *F* in figure 4. The pear-shaped cells in all of these sections were counted, and the total number for each of the six regions 120 μ thick, taken from different regions of the siphon, ascertained. The results obtained are presented in table 9. These results show that there are more of the pear-shaped cells near the middle of the siphon than elsewhere, that the number diminishes from the middle toward the tip and toward the base, and that at the base there are but few. Sections obtained still farther down than region *F* (fig. 4) contain practically no pear-shaped cells.

By comparing the distribution of these cells with the distribution of sensitiveness in the siphon (table 7), it will be seen that photic sensitivity is definitely correlated with abundance of these cells, the region in which they are most abundant being most sensitive. This correlation strongly indicates that the pear-shaped cells are photosensitive.

Further evidence in support of this conclusion will be presented in the following sections.

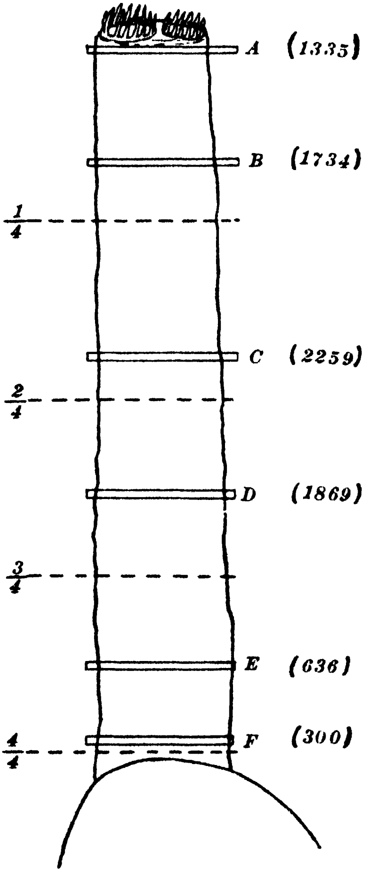


Fig. 4 Drawing of the siphon of Mya, showing the location of the regions sectioned (table 11). Figures in parenthesis indicate the number of pear-shaped cells found in ten adjoining serial sections, each 12 μ thick, taken from each of the regions designated *A*, *B*, *C*, *D*, *E*, and *F*. Dotted lines indicate the points at which the siphon was cut off in ascertaining the distribution of sensitiveness in it.

Form and structure of the pear-shaped cells

The drawings and description here given are based chiefly on a study of silver-nitrate preparations made with a Zeiss mono-objective, binocular microscope, 12.5× compensating oculars, and a 2-mm. apochromatic objective. Gold-chloride preparations and preparations stained with Heidenhain's iron hematoxylin following fixation in Flemming's strong fixative or with Mallory's connective-tissue stain following fixation in chrom-aceto-formol fixative were also studied.

The pear-shaped cells found in the siphon of Mya vary considerably in size and shape (fig. 5). The variation in size extends from 9 to 15 μ in length and from 5 to 10 μ in width. As a rule, the longest cells are also the widest, the length being approximately twice the width. Some are, however, much narrower in proportion to their length (fig. 5, G). Those situated just beneath the epithelial layer vary more than those lying some distance from it.

TABLE 9

Distribution of pear-shaped cells as indicated by the number found in ten adjoining sections from each of six different regions in the siphon

LOCATION SHOWN IN FIGURE 4	NUMBER OF CELLS		TOTAL NUMBER, BOTH SIPHONS
	Excurrent siphon	Incurrent siphon	
A (tip)	353	982	1335
B	512	1222	1734
C	995	1264	2259
D	1024	845	1869
E	478	158	636
F (base)	68	232	300
Total	3430	4703	8133

As a rule, the cells are broadest at the distal end and taper gradually toward the proximal end. Many of the cells are nearly spherical, except for a slight tapering at the proximal end. Most of them, however, are more elongated, being more nearly ovoid. Irregular-shaped cells are found; these are usually embedded in other tissues, and their shape may be due to crowding. Most of the cells have the proximal end bent at an angle of about 45°. This bend in the cell is of considerable importance, as will be explained later.

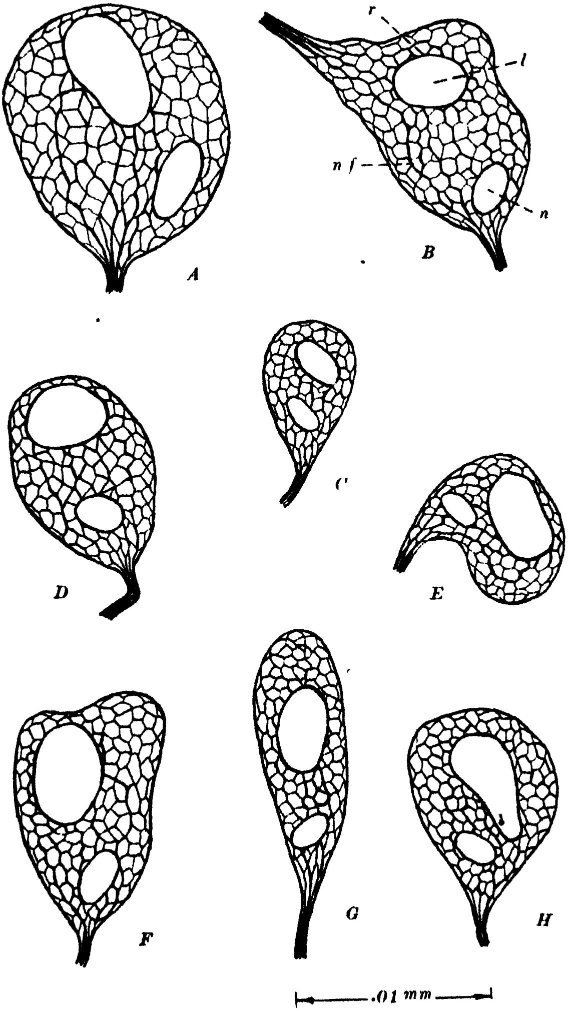


Fig. 5 Camera drawings of longitudinal sections of pear-shaped cells, showing various shapes and sizes. A, cell stained with Heidenhain's hematoxylin; B to H, silver-nitrate technique; B, cell with two fibers. *l*, lens; *n*, nucleus; *nf*, neurofibrils of cytoplasm; *r*, retinella.

As previously stated, these cells are directly connected with the nerves in the siphon. In the silver-nitrate and gold-chloride preparations it can readily be seen that, as a rule, only one nerve fiber enters each cell, although occasionally cells are found which have two fibers entering them (figs. 5, A, and 6, B). These cells are very rare and are only found close to the epithelial layer. The nerve fibers are rather large, as one would expect in cells that possess much nervous activity. Each nerve fiber contains numerous fibrils. It was impossible to follow a fibril from the nerve fiber through the cell, for the fibrils appear to branch and anastomose so as to form a rather dense network extending throughout the entire cell.

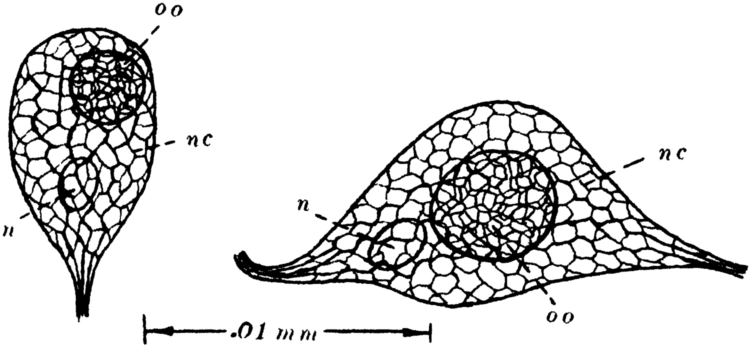


Fig. 6 Camera drawing of pear-shaped cells as seen in side view; silver-nitrate technique. *n*, nucleus; *nc*, neurofibrils of cytoplasm; *oo*, optic organelles.

A rather large nucleus is found, usually lying near the proximal end of the cell. There is no indication of any neural structures connected directly with it. The cytoplasm, aside from the fibrils, appears homogeneous in the silver-nitrate and the gold-chloride preparations, but it has an alveolar appearance in hematoxylin and in Mallory's connective-tissue preparations.

The most conspicuous feature of the pear-shaped cells is a large intracellular structure, lying near the surface, at the distal end. This structure is peculiar to these cells, and is a characteristic by which they can readily be recognized. It varies in size from 5 to 7 μ in length and from 3 to 5 μ in width. The largest ones are not always found in the largest cells. These structures are sometimes spherical in shape, but they are usually elongated, and somewhat tapering or curved (fig. 7); they are always nearly circular in cross-section.

The surface of this structure presents a very dense appearance in silver-nitrate preparations, which on careful examination can be seen to be due to the presence of numerous, rather large, anastomosing neurofibrils. These neurofibrils are connected directly with those which appear to branch from the fibrils that enter the cell. The inner region of this conspicuous structure is composed of a transparent, hyaline substance, which seems differentiated into zones of different density.

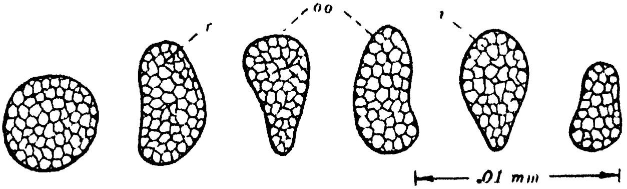


Fig. 7 Camera drawings of several optic organelles, as seen in side view; silver-nitrate technique. *oo*, optic organelles; *r*, retinella.

Hesse ('96) found a somewhat similar structure in the earthworm Lumbricus, which he called a 'Binnenkörper,' and Apáthy ('97), in the leech Pseudobranchellion, which he called a 'Glaskörper.' Apáthy called the cell in which the 'Glaskörper' was found a 'Retinazelle.' He thus suggests that the 'Glaskörper' functions as a lens in a visual organ. Beer ('01), in discussing the 'Glaskörper' of Pseudobranchellion described by Apáthy, refers to it as 'Binnenkörper,' using the term first employed by Hesse. Hess ('25) calls these cells photoreceptors, and furnishes evidence which indicates that the 'Binnenkörper' serves to focus rays of light upon the neurofibrils which surround it; he consequently proposed to designate it 'optic organelle.'

Evidence will be presented in the following section to show that the structures found in the pear-shaped cells of Mya have the same function as the optic organelle found by Hess in Lumbricus. They will also therefore be called 'optic organelles.'

Function of the optic organelle

Small portions of the septum separating the incurrent and excurrent siphons were dissected from the cut-off tips of recently operated animals, placed under the binocular microscope in normal salt solution, and carefully teased with fine needles. In this way some of the pear-shaped cells were loosened from their nerve attachments. Several of these were transferred by means of a pipette and mounted in a hanging-drop of normal salt solution, others were placed on a glass slide and covered with a cover-glass. The cells thus mounted were placed on the stage of a compound microscope and exposed to illumination from below and from the side. In illuminating from below, the condenser of the microscope was removed and the light reflected up with the plane mirror. Lateral illumination was accomplished by means of a Spencer microscope lamp.

Under a magnification of about 300 diameters, definite focal regions of light are clearly seen. These regions are always on the side opposite that at which the light enters. This region is sometimes small and circular in outline and sometimes elongated, forming a band of light which is never of a greater length than the optic organelle. The focal region is always at the surface of the optic organelles, on the neurofibrillar network which surrounds it, irrespective of the direction in which the light enters the cell. It appears, then, that there is a refracting body in the cell which is of such a nature that it brings light to a focus at the surface of the optic organelle, irrespective of the direction. This indicates that the optic organelle is a lens.

The arrangement of the pear-shaped cells, previously described, so as to lie at an angle of about 45° with a perpendicular to the inner wall of the siphon is of considerable importance in receiving light directed into the siphons, for more light enters the optic organelles when they are arranged at this angle than when either perpendicular or parallel to the siphon wall.

If the objective of the microscope is slightly raised or lowered, while light reflected from below is focused on the neurofibrillar network of the optic organelle, then the spot of light changes color. When the objective is raised, the spot becomes reddish in color; when it is lowered, the spot has a bluish tinge at its border. This change in color is a common characteristic due to the refraction of light in lenses, and supports the conclusion that the hyaline interior of the optic organelle functions as a lens.

If the hyaline interior of the optic organelle actually focuses the light on the neurofibrillar network surrounding it as maintained above, the neurofibrillar network is probably photosensitive. If it is, it may be designated a 'retinella.' This is in full accord with the conclusion of Hess ('25) concerning the function of the optic organelle in Lumbricus. If all this obtains, the pear-shaped cells containing the optic organelles are photoreceptors.

RELATION BETWEEN LOCATION AND DISTRIBUTION OF PHOTORECEPTORS AND THE HABITS OF THE ANIMALS

We have seen that the photoreceptors are located near the inner epithelial layer of the siphons. This is where one would expect them, considering the habits of the animal.

Mya in its natural habitat lies embedded in the sand or mud, with the siphon perpendicular to the surface. The outer wall of the siphon is entirely surrounded by the sand or mud, and it would be almost impossible for photoreceptors to function if they were located near this wall. The tip of the siphon is just about on a level with the surface, and when it is open, the inner walls of the siphon are exposed to rays of light from above.

When the animal is disturbed in any way, it suddenly contracts, withdrawing the tip of the siphon and closing the openings. This contraction is sufficient to withdraw the tip of the siphon from immediate reach of enemies. Having withdrawn within its burrow, there is no need for photoreceptors, for any attempt to reach the animal must of necessity disturb the surrounding sand, thus warning the animal through its sense of touch. Before the siphons are extended, they are opened, then they are slowly extended to the surface. This procedure one would expect in animals that have the photoreceptors located on the inside of the siphon, for if they were located in the tentacles or near the outer surface, there would be no need for opening the siphon before extending it.

Piéron ('25) claims that Mya lies buried in the sand and mud during the day, and extends its siphon above the surface of the sand at night to feed. If this is true, the photoreceptors seem to function in keeping the siphon from exposure during the day, when most enemies are active.

Tryon ('82), Lovell ('67), Ingersoll ('87), Kellogg ('10), and others state that the enemies of Mya consist of the walrus, the Arctic fox, cormorants, gulls, crows, etc., and various kinds of fishes. From personal experience nothing is known concerning this except the following: Crows were seen walking along the edge of the water at low tide during the winter season, when food was scarce because of a blanket of snow on the ground; and upon going to the spot where the crows had been, there were found numerous indications of small specimens of Mya recently pulled out of their burrows, their shells crushed, and edible portions eaten. In one instance a medium-sized Mya was found, exposed to the cold air and benumbed by it, with the tender tip of the siphon picked off by a crow. The tracks of the crow were clearly visible in the sand, and the crow itself was seen at that spot a short time before.

If the loss of tips of siphons as described above is a regular occurrence, then the fact that the photoreceptors are distributed along the entire inside of the siphons is of considerable importance to these animals, for with such distribution a portion of the siphon might be removed by an enemy, and the remnant would still function.

RELATION OF PIGMENT SPOTS TO PHOTORECEPTORS

As stated by Ryder ('83) and Sharp ('84), pigment is found on the upper end of the siphon of Mya, both inside and outside, and lines of pigment are found around the base of the tentacles. Ryder claims to have found it in the epidermis and beneath it. In my preparations no pigment was found under this layer. Pigmented cells were found at various places in groups, as stated by Sharp, but these groups were not found in particularly protected places as Sharp contends. They are fairly regularly arranged on the entire surface of the tip of the siphon.

Most previous workers contend that the presence of pigment near the tip of the siphon indicates that there are visual organs in Mya, for in many instances it is well known that visual organs and pigment are closely associated. In my preparations, however, no relation was found between the pigmented areas and the photoreceptors. The pigmented areas are more numerous near the tip and do not extend any considerable distance down along the inside of the siphon, while the photoreceptors are found all along the entire inner wall of the siphon.

The probable value of pigment spots on the siphon of Mya lies not in its function in photoreception, but in its ability to more nearly simulate the background through which the tip of the siphon protrudes. The spotted tip of the siphon closely resembles grains of sand surrounding it, thereby escaping detection and thus affording protection from enemies.

SUMMARY

If Mya is subjected to a sudden increase in illumination, it responds with a marked contraction of the siphon.

The reaction time decreases as luminous intensity increases.

It is shorter with illumination directed into the siphon than in lateral illumination of the same intensity. The inner surface is more sensitive than the outer.

It is shorter when large areas are illuminated than when small areas are illuminated in the same intensity.

It is shorter in small than in large animals.

The siphon is sensitive throughout its entire length. The middle portion is most sensitive, the sensitivity decreasing from the middle toward the tip and toward the base, becoming practically zero at the base.

Regeneration of tentacles on stumps of severed siphons takes place, but there is no regeneration of other tissues.

Eight large nerves, extending the entire length of the siphon and lying closer to the inner siphon wall than the outer wall, surround both the incurrent and the excurrent siphon.

Pear-shaped cells that take on stain for nerve tissue are found beneath the inner epithelial layer in the siphon, but not in other parts of the siphon.

The distribution of these cells in the siphon is closely correlated with the distribution of sensitivity in reference to photic stimulation.

Each of these cells is supplied with a large nerve fiber, and each contains a characteristic structure, which is similar to that found by other investigators in other forms designated 'Binnenkörper,' 'Glaskörper,' and 'optic organelle.'

This structure we will call optic organelle. The inner portion of it is hyaline; it refracts light and focuses it upon a neurofibrillar network, the retinella, which surrounds it.

The optic organelles vary considerably in size and shape, but they always appear circular in cross-section.

The pigmented spots found both on the inside and the outside of the siphon in Mya have no relation whatever with the position and function of the pear-shaped cells. They probably function in simulating the background, thus protecting the siphon against enemies.

The distribution of photoreceptors along the entire inside of the siphons is of considerable importance to these animals, for with such distribution, if a portion of the siphon is removed by an enemy, the remainder can still function.

The photoreceptors found in the earthworm and the visual cells found in the leech appear to be homologous with the pear-shaped cells found in Mya.

BIBLIOGRAPHY

APÁTHY, S. 1892 Erfahrungen in der Benhandlung des Nervensystems für histologische Zwecke. Zeitsch. f. wiss. Mikr., Bd. 9, S. 15–37.

——— 1897 Das leitende Element des Nervensystems und seine topographischen Beziehungen zu den Zellen. Mitth. a.d. zool. St. zu Neapel, Bd. 12, S. 495–748.

BEER, TH. 1897 Die Acommodation des Cephalopodenauges. Pflüger's Archiv., Bd. 67, S. 541–586.

——— 1901 Ueber primitive Sehorgane. Wiener klinische Wochenschrift, Nr. 11, 12, und 13, S. 1–73.

BELDING, D. L. 1909 A report upon the mollusk fisheries of Massachusetts. The Commonwealth of Mass. Special Report of the Mass. Comm. on Fisheries and Game.

BETHE, A. 1896 Eine neue Methode der Methylenblau-Fixation. Anat. Anz., Bd. 12, S. 438–446.

BRONN, H. G. 1862 Classen der Weichthiere, Bd. 3, Abth. I, S. 399.

BROOKS, W. K. 1874 An organ of special sense in the lamellibranchiate genus Yoldia. Proc. Amer. Ass. Adv. Sci., vol. 23, pp. 80–82.

BÜTSCHLI, O. 1886 Notiz zur Morphologie des Auges der Muscheln. Festsch. des naturh.-medic. Vereins zu Heidelberg, Bd. B, S. 173–180.

CARRIÈRE, J. 1885 Die Sehorgane der Thiere. München und Leipzig.

——— 1889 Über Molluskenaugen. Archiv. f. mikr. Anat., Bd. 33, S. 378–402.

CLARK, J. H. 1922 A photo-electric theory of color vision. Jour. Opt. Soc. Amer., vol. 6, pp. 813–826.

DROST, K. 1886 Ueber das Nervensystem und die Sinnesepithelien der Herzmuschel. Morph. Jahrb., Bd. 12, S. 163–202.

DUVERNOY, G. L. 1845 Note additionelle au mémoire sur le système nerveux des Mollusques acéphales bivalves. Comptes rendus hebdomadaires des Séances de l'Académie des Sciences, T. 20, pp. 482–484.

——— 1854 Mémoires sur le système nerveux des Mollusques acéphales, lamellibranches ou bivalves. Mémoires de l'Académie des Sciences de l'Institut de France, T. 24.

FIELD, I. A. 1922 Biology and economic value of the sea mussel (Mytilus edulis). Bull. U. S. Bureau of Fish., vol. 38, pp. 127–259.

FLEMMING, W. 1869 Die haaretragenden Sinneszellen in der Oberhaut der Mollusken. Archiv. f. mikr. Anat., Bd. 5, S. 415–445.

——— 1870 Untersuchungen über Sinnesepithelien der Mollusken. Archiv. f. mikr. Anat., Bd. 6, S. 439–472.

FOLGER, H. T. 1927 Reactions to light by Mya arenaria in relation to the Bunsen-Roscoe law. Anat. Rec., vol. 34, pp. 115–116. (Abstract.)

GANONG, W. F. 1889 The economic mollusca of Acadia. Article I. Bull. Nat. Hist. Soc. of New Brunswick, no. 8.

GEGENBAUR, C. 1874 Grundriss der vergleichenden Anatomie. Leipzig.

GRABER, V. 1883 Fundamentalversuche über die Helligkeits- und Farbenempfindlichkeit augenloser und geblendeter Thiere. Sitzungsb. d. k. Akad. der will. Math.-naturw. Klasse Wien., Bd. 87, Abth. 50, S. 201–236.

——— 1884 Grundlinien zur Erforschung des Helligkeits- und Farbensinnes der Thiere. Prag und Leipzig.

Grube, A. E. 1840 Über Augen bei Muscheln. Müller's Archiv. f. Anat. und Physiol., S. 24–35.

Guyer, M. 1917 Animal micrology. 2nd ed. Univ. of Chicago Press.

Hecht, S. 1919 Sensory equilibrium and dark adaptation in Mya arenaria. Jour. Gen. Phys., vol. 1, pp. 545–558.

———— 1919 The nature of the latent period in the photic response of Mya arenaria. Jour. Gen. Phys., vol. 1, pp. 657–666.

———— 1919 The effect of temperature on the latent period in the photic response of Mya arenaria. Jour. Gen. Phys., vol. 1, pp. 667–685.

———— 1919 An analysis of the relation between the temperature and the duration of a biological process. Proc. Nat. Acad. Sci., vol. 5, pp. 146–148.

———— 1919 The photochemical nature of the photosensory process. Jour. Gen. Phys., vol. 2, pp. 229–246.

———— 1920 Intensity and the process of photoreception. Jour. Gen. Phys., vol. 2, pp. 337–347.

Heidenhain, M. 1895 Cytomechanische Studien. Archiv. f. Entwmech., Bd. 1, S. 473–577.

Hensen, V. 1865 Über das Auge einiger Cephalopoden. Zeitsch. f. wiss. Zoöl., Bd. 15, S. 155–242.

Hess, W. N. 1924 Reactions to light in the earthworm, Lumbricus terrestris L. Jour. Morph. and Physiol., vol. 39, pp. 515–542.

———— 1925 Photoreceptors of Lumbricus terrestris, with special reference to their distribution, structure, and function. Jour. Morph. and Physiol., vol. 41, pp. 63–93.

Hesse, R. 1896 Untersuchungen über die Organe der Lichtempfindungen bei niederen Thiere. I. Die Organe der Lichtempfindungen bei den Lumbriciden. Zeitsch. f. wiss. Zool., Bd. 61, S. 393–419.

———— 1897 Untersuchungen, u.s.w. III. Die Sehorgane der Hirudineen. Zeitsch. f. wiss. Zool., Bd. 62, S. 671–707.

———— 1900 Untersuchungen, u.s.w. VI. Die Augen einiger Mollusken. Zeitsch. f. wiss. Zool., Bd. 68, S. 379–477.

Hickson, S. J. 1880 The eye of Pecten. Quart. Jour. of Micr. Sci., N.S., vol. 20, pp. 443–455.

———— 1882 The eye of Spondylus. Quart. Jour. of Micr. Sci., N.S., vol. 22, pp. 362–370.

Hyatt, A. 1884 The oyster, clam, and other common molluscs. Boston.

Ingersoll, E. 1887 The clam fisheries. Fishery Ind. of the U. S., sect. V, vol. 2, pp. 581–594.

Kalide, G. 1888 Vorläufige Mittheilungen über Studien am Gastropoden- und am Pectenauge. Zool. Anz., Bd. 11, S. 679–683.

Keferstein, W. 1862 Untersuchungen über niedere See-Thiere. IX. Über den Bau der Augen von Pecten. Zeitsch. f. wiss. Zool., Bd. 12, S. 133–136.

Kellogg, J. L. 1892 A contribution to our knowledge of the morphology of lamellibranchiate molluscs. Bull. of the U. S. Comm. for 1890, pp. 389–436.

———— 1899 Life history of the common clam, Mya arenaria. Bull. U. S. Fish Comm., pp. 193–202.

———— 1910 Shellfish industries. New York.

KROHN, A. 1840 Über augenähnliche Organe bei Pecten und Spondylus. Muller's Archiv. f. Anat. und Phys., S. 381–386.

KÜPFER, M. 1916 Sehorgane am Mantelrande der Pecten-Arten. Jena.

LEYDIG, F. 1851 Anatomische Bemerkungen über Carinaria, Firola, und Amphicora. Zeitsch. f. wiss. Zool., Bd. 3, S. 325–332.

——— 1857 Lehrbuch der Histologie der Menschen und der Thiere. Frankfurt.

LOTSY, J. P. 1893 The food supply of the adult oyster, soft clam, clam and mussel. Johns Hopkins University Circulars, vol. 12, pp. 104–105.

LOVELL, M. S. 1867 The edible molluscs of Great Britain and Ireland, with recipes for cooking them. London.

MACHT, D. I. 1918 A pharmacological and therapeutic study of benzyl alcohol as a local anesthetic. Jour. Pharm. and Exp. Therap., vol. 11, pp. 263–279.

MAIER, B. L. 1892 Beiträge zur Kenntniss des Hirudineen Auges. Zool. Jahrb., Abth. f. Anat. und Ontog., Bd. 5, S. 552–580.

MAST, S. O. 1906 Light reactions in lower organisms. I. Stentor coeruleus. Jour. Exp. Zoöl., vol. 3, pp. 359–399.

——— 1907 Light reactions in lower organisms. II. Volvox. Jour. Com. Neur. and Psy., vol. 17, pp. 99–180.

——— 1911 Light and the behavior of organisms. New York.

——— 1916 The process of orientation in the colonial organism, Gonium pectorale, and a study of the structure and function of the eye-spot. Jour. Exp. Zoöl., vol. 20, pp. 1–17.

——— 1922 Eyes in Volvox and their function. Anat. Rec., vol. 24, p. 397. (Abstract.)

——— 1926 Reactions to light in Volvox, with special reference to the process of orientation. Zeitsch. f. vergl. Physiol., Bd. 4, S. 637–658.

——— 1927 Structure and function of the eye-spot in unicellular and colonial organisms. Archiv. f. Protist., Bd. 60, S. 197–220.

MATTHEWS, S. A. 1928 The palps of lamellibranchs as autonomous organs. Jour. Exp. Zoöl., vol. 51, pp. 209–263.

MEAD, A. D., AND E. W. BARNES 1903 Observations on the soft-shell clam (Mya arenaria). Brown University Contributions from the Anatomical Lab., vol. 3. Providence.

——— 1904 Observations on the soft-clam. Rhode Island, 34th. Ann. Rep. of the Comm. of Inland Fisheries.

NAGEL, W. A. 1894 Beobachtungen über den Lichtsinn augenloser Muscheln. Biolog. Centralb., Bd. 14, S. 385–390.

——— 1894 Ein Beitrag zur Kenntniss des Lichtsinnes augenloser Thiere. Biolog. Centralb., Bd. 14, S. 810–813.

——— 1896 Der Lichtsinn augenloser Thiere. Jena.

PACKARD, A. S. 1889 Zoölogy for high schools and colleges, pp. 222–227. New York.

PARKER, G. H. 1919 The elementary nervous system. Philadelphia.

PATTEN, B. M. 1915 A device for projecting a small spot of light suitable for exploring photosensitive areas. Science, N. S., vol. 41, pp. 141–142.

PATTEN, W. 1886 Eyes of molluscs and arthropods. Mitth. a.d. Zool. Stat. zu Neapel, Bd. 6, S. 542–756.

PELSENEER, P. 1891 Contribution à l'étude des Lamellibranchs. Archiv. de Biol., T. 11, pp. 147–312.

PIÉRON, H. 1925 La loi de l'excitation lumineuse chez Mya arenaria. C. R. Soc. de Biol., T. 93, pp. 1235–1238.

——— 1926 Les lois de l'excitabilité lumineuse des Lamellibranches. Recherches sur la loi de variation des énergies liminaires chez Mya arenaria. Bull. de la Station Biologique d'Arcachon., T. 23, pp. 1–32.

POLI, J. X. 1795 Testacea utriusque Siciliae eorumque historia et anatome tabulis aeneis illustrata, vol. 2, p. 107. Parmae. (Reference from Sharp, '83.)

RAWITZ, B. 1887 Das centrale Nervensystem der Acephalen. Jenaische Zeits. f. Naturw., Bd. 20, S. 384–460.

RETZIUS, G. 1892 Biologische Untersuchungen von Prof. Dr. Gustaf Retzius Neue Folge, Bd. 4, S. 1–70.

RYDER, J. A. 1883 Primitive visual organs. Science, O. S., vol 2, pp 739–740

——— 1885 On the green coloration of the gills and palps of the clam (Mya arenaria). Bull. U. S. Fish Comm., vol. 5, pp. 181–185.

——— 1885 On the rate of growth of the common clam, etc. Bull. U. S. Fish Comm., vol. 5, pp. 174–176.

——— 1889 The byssus of the young of the common clam (Mya arenaria). Amer. Nat , vol. 23, pp. 65–67.

SCHREINER, K. E. 1896 Die Augen bei Pecten und Lima. Bergens Museums Aarbog., Nr. 1, S. 1–51.

SHARP, B. 1883 Visual organs of Solen. Science, O S., vol. 2, p. 692.

——— 1884 On the visual organs in lamellibranchiates. Mitth a.d. Zool. Stat. zu Neapel, Bd. 5, S. 447–470.

SIEBOLD, C. T. V 1848 Lehrbuch der vergleichenden Anatomie der wirbellosen Thiere. Berlin.

SIMROTH, H. 1876 Ueber die Sinneswerkzeuge unserer einheimischen Weichthiere. Zeitsch. f. wiss. Zool., Bd. 26, S. 226–349.

TALIAFERRO, W. H. 1920 Reactions to light in Planaria maculata, with special reference to the function and structure of the eyes. Jour. Exp. Zool., vol. 31, pp. 59–116.

THIELE, J. 1889 Die abdominalen Sinnesorgane der Lamellibranchien. Zeitsch. f. wiss. Zool., Bd. 48, S. 47–59.

——— 1890 Ueber Sinnesorganeder Seitenlinie und das Nervensystem der Mollusken. Zeitsch. f. wiss. Zool., Bd. 49, S. 385–432.

TRYON, G. W. 1882 Structural and systematic conchology. Philadelphia.

VERRILL AND SMITH 1874 Report upon the invertebrate animals of Vineyard Sound and adjacent waters. Rept. U. S. Com. Fish and Fisheries on the Cond. of the Sea Fisheries of the South Coast of New England in 1871 and 1872.

WENRICH, D. H. 1916 Notes on the behavior of bivalve molluscs to changes in light intensity: Image formation in Pecten. Jour. Animal Behavior, vol. 6, pp. 297–318.

WHITMAN, C. O. 1889 Some new facts about the Hirudinea. Jour. Morph., vol. 2, pp. 586–599.

WHITMAN, C. O. 1893 A sketch of the structure and development of the eye of Clepsine. Zool. Jahrb. Abth. f. Anat. und Ontog. der Thiere, Bd. 6, S. 616–625.

WILL, F. 1844 Über die Augen der Bivalven und Ascidien. Froriep's Neue Notizen a.d. Gebiete der Natur- und Heilkunde, Bd. 29, S 81–87, 99–103.

WILLEM, V. 1891 Sur les perceptions dermatoptiques. Bull. scientific de la France et de la Belgique, T. 23, pp. 329–346.

WOODWARD, S. P. 1880 A manual of the Mollusca. London.

PLATE 1

EXPLANATION OF FIGURES

8 Photomicrograph of a longitudinal section through the tip of the siphon of Mya, perpendicular to the septum; silver-nitrate technique. *c*, cuticulum; *es*, excurrent siphon; *ie*, inner epithelial layer; *is*, incurrent siphon; *n*, nerve; *oe*, outer epithelial layer; *rp*, region of photoreceptors.

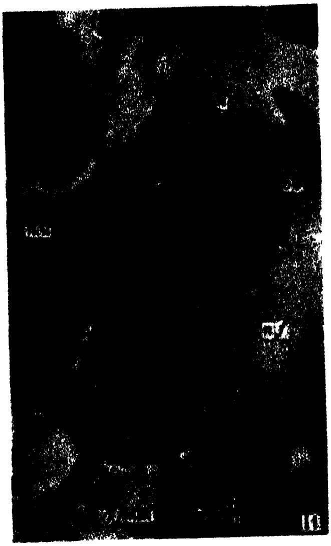
9 Photomicrograph of a cross-section through tip of Mya siphon, about 1 cm. from the tentacles; silver-nitrate technique. Labels same as for figure 8.

10 Photomicrograph of a portion of the septum of Mya, in the region of the photoreceptors; silver-nitrate technique. *ie*, inner epithelial layer; *n*, nerve fibers; *p*, photoreceptors.

11 Photomicrograph of two photoreceptors shown in figure 10; silver-nitrate technique. *n*, nerve fiber; *nf*, neurofibrils; *nu*, nucleus; *oo*, optic organelle.

9

10


11

THE NEUTRAL-RED REACTION

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FOUR TEXT FIGURES AND FOUR COLORED PLATES (NINETY-THREE FIGURES)

AUTHOR'S ABSTRACT

A general survey of the reaction of neutral red upon a selected series of living animal organisms is made, and the rationale indicates that neutral red is an indicator of proteolytic enzyme action, whether this be intra or extracellular. Cellular hydrolytic and cellular synthetic systems are thus manifested. The neutral-red reaction also reveals the course of assimilation and secretion in organisms, the nature of fertilization, and the beginning of embryological differentiation.

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PROBLEM, METHODS, AND MATERIALS

Shipley, in 1919, concluded:

> The granule which stains intra vitam in the cell body is then the equivalent of the protozoan food vacuole and since the amicrons of the semi-colloidal dyes are stored in it, we have in benzidine vital staining an index and a demonstration of a feeding and storage reaction on the part of the cells of warm blooded animals.

Neutral red was recognized as the least toxic of the vital dyes.

Marston, in 1923, showed that the azine dyes, especially neutral red and Janus green, have an affinity for enzymes, both in the test tube and in living bodies. They concentrate upon the surface of the enzyme, precipitating it: "The power of the azine and azonium bases to remove completely the proteolytic enzymes from solutions, indicates that a direct union of the enzyme and the precipitating base is established."

Parat, since 1925, has been postulating that in all living animal cells there is a vacuome system colorable by neutral red and that this is the equivalent of the Golgi system in fixed material.

From the time of Ehrlich, 1894, to the most recent papers of Dawson ('29), Covell and Scott ('28), etc., neutral red has been widely used as a vital dye. No comprehensive theories as to its significance have been advanced other than the above-mentioned apparently widely divergent views.

Are these views, nevertheless, harmonious and may a consistent and universal significance be attributed to the neutral-red reaction?

A cytological problem of such fundamental physiological import must be attacked by vital methods interfering in no way with the activities of the organism. This requirement the neutral-red reaction fulfills, for it can be shown that the dye is not toxic, but can be assimilated and eliminated readily by cells, leaving them unimpaired. Problems relating to various manifestations of the neutral-red reaction resolve themselves in the following topics:

I. The cellular hydrolytic system
II. The cellular synthetic system
III. Intra- and extracellular digestion
IV. Elective staining and secretion
V. Development of the egg and differentiation
VI. Development of the sperm and fertilization
VII. The morphology of the neutral red bodies and their relation to the Golgi apparatus and the mitochondria

The material used is listed:

Sarcodina:
  Amoeba proteus
  Amoeba verrucosa
  Amoeba polypodia
  Amoeba radiosa
  Amoeba limax
  Amoeba dubia
  Amoeba guttula
  Hyalodiscus
  Hyalosphenia
  Arcella vulgaris
  Nuclearia simplex
  Actinophrys sol
  Actinospherium
  Heterophrys
  Clathrulina
Mastigophora:
  Mastigamoeba longifilim
  Astasia trichophora
  Notosolenus
  Chilomonas
  Euglena
Infusoria-Ciliata:
  Spirostomum
  Urocentrum
  Colpidium
  Loxophyllum
  Paramecium multimicronucleata
  Paramecium caudatum
  Paramecium aurelia
  Blepharisma laterita
  Stentor polymorphus
  Stylonichia
  Histrio
  Euplotes
  Vorticella
  Trichodina
Suctoria:
  Acineta
Coelenterata:
  Hydra vulgaris
  Microhydra
Turbellaria:
  Stenostomum tenuicauda
  Planaria maculata
  Planaria simplex
Rotatoria:
  Brachionus urceolaris
  Philodena
Gastrotricha:
  Chaetonotus maximus
Annelida:
  Chaetogaster langi + limnaei
  Aeolosoma
Mollusca:
  Limax palustrina
  Physa
Crustacea:
  Cladocera: Daphnia pulex
  Copepoda: Cyclops
  Ostracoda: Cypris virens
Insecta:
  Culex larvae
  Dragon-fly nymphs
  Grasshopper blood elements
  Grasshopper egg strings
  Grasshopper testicular follicles
Amphibia:
  Amblystoma: axolotl larvae
  Rana eggs and tadpoles
Mammalia:
  Human blood corpuscles

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THE CELLULAR HYDROLYTIC SYSTEM

Shipley ('19) describes Protozoa and chick tissue-culture cells alike taking up such substances as neutral red, brilliant cresyl blue, carmine, carbon, bacteria, algae, fat-droplets, free pigment, and other materials of the medium into an identical series of vacuoles. There is never one type of vacuole which takes only dyes, another carbon particles, another bacteria. When two or more of these substances are presented to either Paramecium or clasmatocyte simultaneously or in succession, they are similarly phagocytosed, appearing in the same vacuoles.

In various amoebae and ciliates it is possible to watch ingesting of dyes and food particles, as in some invertebrate (latest reference, Goodrich, '28) and vertebrate cells (Sabin, '25). In other Protozoa, as in mammalian nerve cells (Covell and Scott, '28), pancreas cells (Covell, '28), no vacuole formation is ever noted and the dye is imperceptibly taken up by vacuoles either previously or not visible. For most Protozoa the term 'food vacuole' is used descriptively, but for others and for metazoan cells this meaning is usually not even implied. One object of this investigation is to determine the function of neutral-red bodies wherever they may be found.

Some doubt is often expressed as to the toxicity of neutral red. Wilson ('27) studied the effect of various makes and dilutions of the dye upon Paramecium and concluded that up to 1:100,000 his dilutions of all forms were toxic within a short time, the maximum time for the greatest dilution and best make being about five hours. King ('26), demonstrating the Golgi apparatus in Anophophyra with neutral red, reports that the organisms died before the Golgi could be stained. Parat and Painlevé ('26) recommend only one type of neutral red suitable for the study of the vacuome. Child and Deviney ('26) describe only morbid conditions in stained Paramecium, and Shapiro ('27) likewise uses very toxic solutions.

Bowen ('28) recommends studying neutral-red preparations made by his methods quickly, for: "Such preparations are always short lived and their study must accordingly be completed at once without possibility of later recourse for comparison or critical examination." On the other hand, Sabin, Cunningham, and Doan ('25) say, "Neutral red reactions are so little harmful that they do not inhibit certain activities like cell division and motility for considerable periods of time."

On the whole, however, this vital reagent has been used with less care and experimentation than is often accorded histological stains. It is at this point that the author wishes to suggest a more critical technique. Neutral red can be and should be used in dilutions which are not toxic.

A proper dilution and perhaps make of the stain must be found for each form studied, so that instead of an organism being stained once and studied hastily, it may be stained over and over again so that all phases of the assimilation of the dye, the details at the height of staining, and the gradual elimination may be observed carefully. Or it may be desirable to keep organisms continuously stained over a period of days or weeks. Where the control organisms survive, the experimental individuals survive when neutral red is carefully used. In this study only a Grübler stain was used. A strong solution is made up by adding 10 mg. to 200 cc. of spring water, which gives a bright cherry color. This solution may be suitably diluted and used immediately the dye has gone into solution.

Protozoa are most suitable material for learning how to handle the stain. A drop of culture is placed upon a greaseless no. 0 cover-slip and a small pipette or needle wet in neutral-red solution is touched to the drop and the preparation is inverted over a depression slide, sealed down with a film of water, and immediately placed under observation. (Shapiro's method of allowing organisms to become compressed by a cover-slip upon a flat slide has never been used in this study.) If any of the organisms have taken the stain so quickly, the preparation may be discarded, unless one wishes to study cell morbidity. The instantaneous reaction of active Protozoa and insect blood cells, etc., is truly remarkable. It is invariably fatal.

At the least, two or three minutes should elapse before one can see the faintest tinge of color in the vacuoles of the organisms present. Some amoebae, various Mastigophora, and some of the smaller ciliates easily withstand a strength of the stain that can be visibly concentrated this quickly. For Paramecium, Stentor, and Vorticella, however, fifteen minutes should elapse before the stain becomes brilliant in the vacuoles. After experimentation with several drops has shown that the dye is of proper dilution, the preparation may be more permanently sealed with a warmed vaselin pencil and labeled as to time of staining, approximate dilution, etc. It may now be handled just as similarly prepared tissue cultures are. Amoebae, especially, do well under these conditions, remaining in good shape from six to eight weeks. The cover-glass is lifted when it is desired to restain.

For the most part, Protozoa have been kept in small cultures, 50 to 100 cc., to which the dye is added, accurately measured by means of a 1-cc. syringe graduated into hundredths. The dilutions used range from 1:1,000,000 to 1:15,000,000. These dilutions are practically invisible in the faintly colored culture fluids, yet they produce as brilliant staining in the vacuoles of the organisms as do greater concentrations. The 1:1,000,000 dilution proves toxic for some forms, but the remainder of the range has proved altogether non-toxic.

Paramecium and Vorticella behave similarly in neutral-red solutions and as they excel in being favorable organisms for observation of food-vacuole formation, they may be described together. A 1:10,000,000 solution is chosen as the most ideal dilution. Samples of the cultures are made into hanging-drop preparations frequently, but an hour or more on the microscope stage with the cover-glass well sealed with water does no harm.

Just after the addition of the dye, it will be noted, if the as yet unstained organisms are compared with the controls, that there is a just perceptible increase in the rate of vacuole formation in the experimental animals. The cilia along the base of the gullet or cytopharynx force water and food particles into the fundus of the gullet until the pendant-like structure forming at last, like a heavy drop, flows away connected with the gullet by a long, rapidly narrowing canal which soon becomes invisible.

It seems quite certain that this newly formed vacuole and its slender connecting canal are an invagination of the gullet, its walls, therefore, of ectoplasm and its interior a small column of the external medium into which large and small particles have been sifted. Watching this process of vacuole formation repeated innumerable times at a magnification of 1500, one cannot hold to any of the following theories: that a preformed vacuole received food particles (Shipley, '19): that the food particles are received into the cytoplasm and a vacuole forms about them later (Minchin, '17; Nirenstein, '05; Lewis and Lewis, '24); that cytoplasmic constituents such as mitochondria could be included in the vacuole (Horning, '28; Nirenstein, '05): that the vacuole is in any way of entoplasmic origin (Minchin, '17); that food particles pass through a membrane in entering into the interior of the cell (Harvey, '11; Wenyon, '26).

Instead, the food vacuole in these ciliates is simply a bit of the outside enclosed about by a portion of ectoplasmal membrane and sunk into the cytoplasm of the cell. Greenwood ('94) and Maier ('03) give figures showing this relationship clearly. So far, it is exactly the same condition as is found in Amoeba: "in the formation of each vacuole a sheet of plasmalemma is taken in. This forms a limiting membrane which probably persists until the vacuole disintegrates" (Mast, '26).

From a few seconds to a few minutes after the formation of such an enclosure, a faint pink blush spreads over the vacuole. Meanwhile another drop forms and pulls away, the long canal appearing momentarily. Both ends of the drop are pointed, giving it a spindle shape, with nothing visible at the distal end and the narrow canal at the proximal end. Minchin ('17), noting this phenomenon, gives the following mysterious explanation:

> The vacuole is at first immured in a thin layer of less fluid protoplasm, doubtless as the effect of contact with water; consequently the vacuole is not at first circular, but often spindle-shaped in its contours; it soon, however, assumes a spherical form, indicating that its protoplasmic envelope has become liquefied.

The movement of the newly formed vacuole as it appears to be pulled down into the cytoplasm results, without actual contact, in the slight displacement of its immediate predecessor. The persistence of a flexible, but slightly resistant connection between the two vacuoles is strongly suggested.

So perhaps six or seven vacuole formations are observed, each one pushing the others gently about and each one assuming the pink blush. Meanwhile the first one formed during the observation has become more brilliant, with perhaps the acid red or the neutral brick color. (Yellow vacuoles have not been noted in either of these forms.) Furthermore, those vacuoles already formed when observation was begun and therefore existent before the stain was added to the medium have likewise assumed a red color. How does neutral red get into these vacuoles? In those formed after the stain is added it seems that the dye is phagocytosed with food particles. But while these vacuoles are engulfing stain, vacuoles far to the anterior end of the organism with no opportunity for phagocytosis become even more brilliant than the newly formed vacuoles.

Vorticella presents some suggestive clues to this phenomenon. When all of the vacuoles of the body become stained, it is noted that the rate of vacuole formation, instead of being slightly increased, begins to decrease rapidly until new vacuoles are very slowly formed. Then these cease altogether for a time; the cilia of the gullet reverse their movements and what now appears at first glance to be a new vacuole is an old one which has opened into the gullet and the contents of which are being swept out. Another vacuole follows, smoothly drawn up to the same point in the gullet, opens into the wall with no sign of a membrane rupture, and discharges its contents. As one by one these vacuoles draw up and open out, it is clear that they are doing so in definite order. The oldest vacuoles are first. It is often possible to determine while one is emptying which vacuole will be next. The means by which these vacuoles are drawn to the gullet is imperceptible. There is no visible canal as at vacuole formation. The impression that the opening of a vacuole does not involve a membrane rupture is strengthened by the shaping of the vacuole as it is drawn into the gullet. The perfect sphere slowly gives way to the oval or spindle outline, and the manner in which the flowing vacuole membrane opens into the gullet is the exact reversal of the canal closure in the formation process.

Such evidence leads one to consider carefully the words, "the vacuole system of the organism." May this be a true system continuous from beginning, mouth, to end, anus, and is the whole system an enclosure of the external in the same way that the alimentary tract of any metazoan, an annelid for instance, is an enclosure so that the lumen is in the body but not a fundamental part of the body?

Greenwood ('94), Nirenstein ('05), and Shapiro ('27) show the definite paths of food vacuoles through the cytoplasm from formation to defecation, but they do not suggest any connection of the vacuoles. No fixation or staining methods have ever demonstrated connections, very few methods, for that matter, preserving the food vacuoles themselves. Evidence for the continuity of the system rests only upon the following facts:

1. A slender canal connects the gullet with the newly formed vacuole.

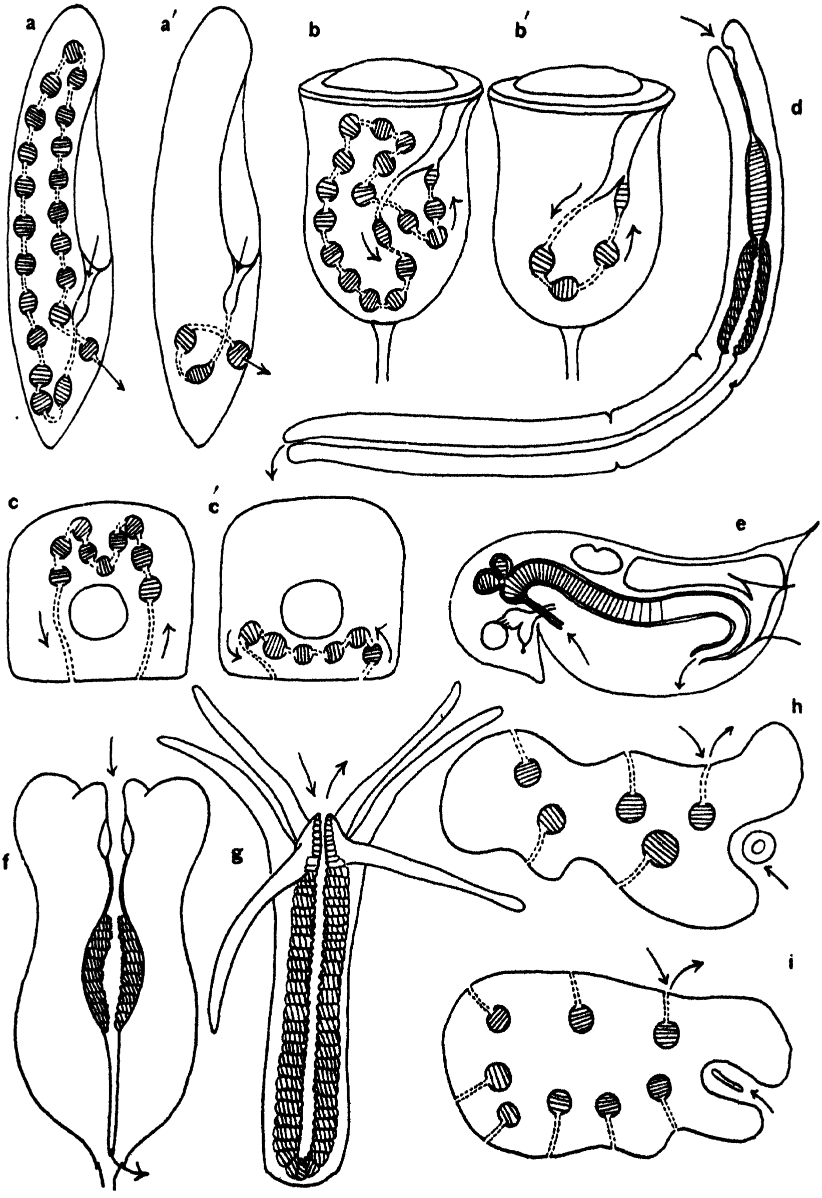


Figure A

2. No single vacuole is observed to move independently of the other vacuoles.

3. The progress of vacuoles through the cytoplasm is such a definite, orderly course as not to be explained altogether as a result of protoplasmic streaming.

4. The systematic drawing up of the vacuoles in defecation is without other explanation. Under the initial stimulation of neutral red it is quite clear, at least in Vorticella, that each vacuole of the system is emptied in correct succession, although the ordinary digestive period has by no means been completed.

5. When dilute neutral-red solutions are used, only the food vacuoles receive the stain just as they receive other colloidal particles, yet the vacuoles formed previously to the addition of the stain acquire color fairly quickly. On the contrary, when larger particles such as carmine and India ink are introduced they can be found only in the vacuoles formed subsequent to the addition of these substances. There may, then, be connecting canals admitting neutral red to the whole system; these canals of such minute size that only the finest of colloidal particles pass through?

Thus the idea of a digestive system in ciliates, single cells of more or less fixed shape, comparable to the digestive systems of many metazoan organisms presents itself as a possible interpretation of recognizable phenomena, and forms a hypothesis to which many of the subsequent data presented in this investigation lend color.

Fig. A Diagrams of various forms illustrating two possible types of digestive systems. Those parts of the systems staining with neutral red are cross-hatched. Parts which cannot be demonstrated are in dotted lines. a. Paramecium, non-amoeboid type. Condition x, normal active hydrolysis. a′. Condition y, pathological, a result of hyperstimulation. b. Vorticella, non-amoeboid type. Condition x, normal active hydrolysis. b′. Condition y, pathological, a result of hyperstimulation. c. Hypothetical vertebrate fixed-tissue cell: e.g., thyroid cell, non-amoeboid type. Condition x, active hydrolysis (Champy, '12). c′. Condition y, pathological (?). d. Annelid worm, non-amoeboid type, but some amoeboid digestion. e. Arthropod, non-amoeboid type. f. Rotifer, non-amoeboid type, with amoeboid digestion. g. Coelenterate, amoeboid type. h. Amoeba. i. Leucocyte, amoeboid type.

In contrast to cells with perhaps a single, continuous digestive tract, Amoeba presents the discontinuous type of digestive apparatus. Food may be enclosed about by the plasmalemma at any point on the body surface instead of at a specialized point, and it may be ejected within a few minutes by the reopening of this plasmalemma to the surface again, or retained for many days (Schaeffer, '16).

Amoeba proteus is a cell with comparatively large and distinct food vacuoles. Arcella, rotifers, flagellates, and other fair-sized forms are ingested, usually with very little water about them, so that, especially with a quiet form like the Arcella shell, it requires the highest magnification to see the vacuole lumen and the limiting membrane. Were it not for the large size of these structures and also comparison of vacuoles about quiet food forms with those much larger vacuoles about very active ingested materials, the observations of Lewis and Lewis ('24) for metazoan phagocytes might hold: "that ingested granules enter directly into the homogeneous cytoplasm and not into preformed vacuoles and that vacuoles do not form about such particles coincident with their entrance into the cell."

On the contrary, it is as difficult to conceive of ingestion without vacuole formation as it is to conceive of preformed vacuoles. Amoeboid phagocytosis involves the formation of food cups (Schaeffer, '16, and Mast, '26), and were it possible to observe all amoeboid-like phagocytosis with the ease and clarity possible in an enormous cell like the proteus such a conception of the process of ingestion would be universally recognized as fundamental.

In A. proteus newly formed vacuoles are slow to begin digestion and even killing of the ingested organism is often long delayed. A rotifer, for example, may remain an hour in a vacuole before movement of the mastix stops and before any trace of neutral red appears in this vacuole lumen. When color does appear, it is as the Lewises describe it, "as a slight halo" about the ingested matter, and, to be sure, it brings out the lumen and limiting membrane much more clearly than before staining, but it has nothing to do with vacuole formation.

The existing and active digesting vacuoles in proteus stain within a moderate interval compared with the rapidity with which actively digesting Paramecium vacuoles stain—within ten minutes in a 1: 1,000,00 dilution and somewhat longer in greater dilutions. The vacuole membrane is not conspicuous; the fluid of the lumen is usually faintly red or pink, and it is upon the food mass that the neutral red concentrates, producing, for the most part, a dense, rather dull brick red. There are occasional orange and yellowish bodies, depending, seemingly, more upon the type of food than the age of the vacuoles.

How does neutral red get into vacuoles formed before the addition of the stain? When food cups sink into the cytoplasm, a narrow canal is indicated for a short time connecting it with the outside (see Grasse-Allermann's '09 figures). The general opinion is that this does not persist and that the food vacuole floats freely in the cytoplasm (Shipley, '19). If the cup closes completely and loses all connections, how are the following phenomena to be explained? A. proteus often swallows very active organisms which thrash about for a considerable period after the vacuole is fully formed and often at a considerable distance from the cell boundary. Occasionally such an organism is seen to wriggle its way out of the vacuole and to escape from the amoeba. There is no sign of a membrane rupture at the vacuole wall nor at the wall of the cell. Aside from this more or less definite evidence that the food vacuole retains connection with the outside, it is difficult to presume that a foreign organism could rupture two membranes and plow through the groundwork of the cytoplasm without injuring the cell.

Then there is the matter of defecation. Schaeffer reports Amoeba ingesting and ejecting the same carmine granule five or six times in succession within a short time. Or food may be retained for many days, when all that has not been made soluble is cast out, for example, the Arcella shell. Combing protozoological literature elicits no more definite statements as to the manner of egestion than that material is 'cast out' or 'thrown out' or 'the vacuole disintegrates.' An absolutely disconnected vacuole is always assumed.

Two papers, however, H. J. Child ('26) and Kofoid and Swezy ('26), show unmistakably that the vacuole may remain connected with the outside, for in the extrusion of food material they see "a canal which is continuous with the vacuole from which the extrusion arises, and which contains, surrounding the strand, the usual clear fluid of the vacuole" (Child, '26).

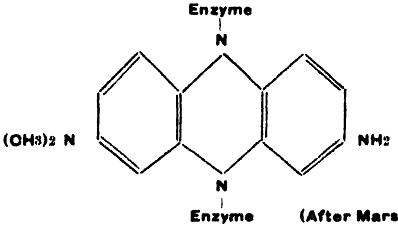
Thus the digestive system of Amoeba may be thought of as a discontinuous series of vacuoles of invaginal origin, in contrast to the continuous system of like origin in cells of more constant form. The vacuoles may retain their ectoplasmal nature in both the Amoeba and the ciliate types throughout their history, although the following opinion as expressed by Schaeffer seems to be somewhat generally held: "This process may be regarded as a change of the ectoplasm lining the vacuole, to endoplasm, which now fills up most of the space occupied by the water, according to the suggestion first advanced by Wallich (1863)." Why must one membrane be arbitrarily exchanged for another; what is the mechanism of such an exchange; and what is the nature of the membrane when the vacuole is again opened to the outside? The suggestion involves difficulties.

Much simpler is the conception that the food vacuole is an ectodermal enclosure of a small volume of the external medium containing usually some valuable particulate matter and that it remains essentially unchanged throughout its duration in the organism. If the contained matter be suitable, enzymes are secreted into the vacuole and dissolved substances are prepared to be taken into the cytoplasm. Sucked dry, and this is usually literally true, the vacuole opens again to the outside in the same manner as it was formed. No raw material nor useless nor poisonous matter ever normally enters the cytoplasm or passes a cell membrane. The Paramecium and the Amoeba each may ingest glass, carbon, carmine, soil, and heavy cellulose walls in the same manner as the child may swallow pennies, cherry pits, and mud pies. The penny never passes a body membrane.

As for the problems of the handling of digestible substances, neutral red plays a significant part in demonstrating the relationship of the vacuole to the cytoplasm. The existing vacuoles in any cell placed in a suitable dilution of neutral red will slowly take up the stain. No other part of the cell shows any trace of it. If the vacuoles contain large food masses, as in A. proteus, or clumps of small particles, as in Paramecium, the particulate matter stains more deeply than the fluid of the vacuoles. If the food matter be so finely divided as to be of colloidal nature, as in many flagellates, it seems to be uniformly distributed in the vacuole and the whole structure stains homogeneously. A vacuole newly formed in the staining medium does not so quickly assume color as do those actively digesting vacuoles. (No diminution of the amount of staining fluid is responsible for this, as staining of new vacuoles may continue for five to six days for some organisms in dilutions of 1:10,000,000.)

This suggests a reasonable explanation: All vacuoles of the cell are permeable to the staining particles whether they belong to the continuous or discontinuous type of digestive system. In those which have been formed for some time and in which digestive processes are going on the dye is rapidly concentrated in the fluid or enzyme part of the vacuole, appearing most dense where the enzyme is most concentrated. In newly formed vacuoles there is fluid, but it is the fluid of the medium in which neutral red is so disperse as to be invisible. (Even in bulk a 1:10,000,000 solution is invisible.) As this fluid is absorbed into the cytoplasm it is thought to be replaced by enzymes. The initial secretion is demonstrated by the faint halo or pink blush noted in all newly formed vacuoles. Progress in digestion increases the density of the blush to brilliancy. The resulting color may be acid, neutral, or alkaline, and this color may persist throughout the history of the vacuole or it may change, as it commonly does from the acid to the alkaline range in the progress of digestion. Some cells have only acid vacuoles (ovarian supporting cells in Daphnia), or only neutral vacuoles (neutral phagocytic system in Daphnia), or only alkaline vacuoles (alkaline phagocytic system in Daphnia), while other cells show more or less rapid changes from acid to neutral and alkaline vacuoles so that all three exist at times in the same cell (developing grasshopper blood cells).

What are enzymes that their presence might be visibly demonstrated by a dye? Bayliss ('23) says, "The facts that enzymes are in the colloidal state and that their action takes place on the surface of the enzyme phase are now generally admitted." What is neutral red that it may be concentrated upon the surfaces of enzymes? Neutral red is the hydrochloride of dimethyldiaminotoluazine, and since Robertson's work, in 1907, and Holzberg's, in 1913, it has been known that azine dyes precipitate enzymes. Marston, in 1923, investigated the nature of this precipitation and found that enzyme preparations of the type of pepsin, trypsin, and erepsin combine with neutral red, thus inactivating these proteolytic enzymes quantitatively and also precipitating any lipolytic and diastatic substances found in conjunction with them. This work is sustained by Epstein and Rosenthal in 1924. Cytologists in general have disregarded this knowledge, although Marston states: "As the azine and azonium bases are colored substances the occurrence of the proteolytic enzymes



(After Marston, '23)

Fig. B The combination of dimethyldiaminotoluazine with proteolytic enzymes.

in the cell may be demonstrated by means of their selective staining reactions."

The importance, cytologically and physiologically, of being able to demonstrate the apparatus of digestive processes and the presence of digestive substances in cells cannot be overestimated. For, although the action of neutral red upon enzymes is a precipitating and therefore partially inactivating effect (30 per cent), it is quantitative, and when small quantities are used its only immediate effect seems to be the slight stimulation of further enzyme production. Such stimulation is manifest in increased vacuole formation in ciliates, increased division in Amoeba, and even in accelerated spermatogenesis as noted by Voïnov in 1927. Generations of Protozoa may be produced in dilute neutral red; conjugation is frequently noted in Paramecium and in high percentages—approximately 80 per cent in P. multimicronucleata; turbellarian worms increase to an extent as to become a nuisance in mixed cultures; in fact, all of the organisms listed in the introduction to this paper respond favorably to staining with neutral red, with the exception of Rana tadpoles. And in all of the organisms throughout the invertebrate series, at least, the manifestation of neutral red indicates the apparatus for, and the nature of, digestion.

Now it has always been difficult to conceive of proteolytic enzymes existing in an active form within the cell without autocytolysis, although such enzymes have long been known. Mouton, in 1901, extracted from Amoeba an active proteolytic diastase much like trypsin, but active in a range from the distinctly alkaline to the weakly acid. Dochez, in 1909, describes the enzyme obtained from polymorphonuclear leucocytes as resembling trypsin from the pancreas, but unlike trypsin, which exists in the activated form only outside of the cell, the polynuclear enzyme exists within the cell in an active state. This enzyme, called by Opie ('10) 'leucoprotease,' is purified by precipitation with alcohol, may be preserved indefinitely, is destroyed between 70° and 75°C., and acts in an alkaline or in a neutral medium, being inhibited by acid.

Jochmann ('08) has demonstrated 'lymphoprotease' from the mononuclear phagocytes of lymph nodes, this enzyme, like pepsin, acting in an acid medium, being inhibited by alkali. Opie ('10) reviews the whole subject of the enzymes of various tissue cells begun by Metschnikoff in 1905. It is clear that each cell has a more or less specific type of enzyme existing within itself in an active state.

Now with the above-suggested theory as to the invaginal nature of the cellular digestive system, it is at once evident that it may not be necessary to think of these hydrolytic enzymes existing within the cytoplasm of the cell. A vacuole, or a connected series of vacuoles, which is an ectoplasmic enclosure of a small volume of the external medium, is then not a true cytoplasmic constituent. It becomes a lumen for the enclosure of food matter while subject to digestion. It is therefore not necessary to conceive of a killing agent for bacteria and rotifers which Amoeba ingests, nor for a hydrolyzing enzyme which will subsequently digest these substances, as in the active state in the cytoplasm. The cell enzymes may be poured into watery vacuoles and there activated into hydrolytic agents, just as pepsin, trypsin, and all similar hydrolytic substances are activated in digestive lumina. The proteolytic process may be extracytoplasmic in all animal life.

With these conceptions in mind—that the neutral-red reaction is a manifestation of enzyme action, and that cellular proteolytic activity may take place in vacuoles of a continuous or discontinuous type of invagination—much evidence presents itself in support.

The author regards the biochemical evidence offered by Robertson, Holzberg, Marston, and Epstein and Rosenthal as to the relationships between azine and azonium compounds and proteolytic enzymes as merely confirmative of the striking manifestations of these relationships in vivo. This paper will demonstrate that wherever in both protozoan and metazoan forms proteolytic enzymes are known to exist in active form, there will be found azine-dye staining. Wherever it is known positively that proteolytic enzymes do not exist and cannot exist even under experimental conditions, due to various inactivating or 'anti-enzyme' effects, there azine staining can never be demonstrated. The fact that the biochemists offer a quantitative aspect of this phenomenon so adequately demonstrated by living material is promising for future work.

That there is any absolutely specific relationship between azine dyes and enzymes is not at all presumed in this paper. That there may be a specificity between enzymes and a certain very definite chemical ring common to both azines and proteins, as Robertson ('28) postulates, is interesting, but not necessarily essential in the explanation of the phenomena described for in-vivo material. That proteolytic enzymes combine with other things than azine dyes and proteins is well known. Hsü ('24) finds trypsin adsorbed by filter-paper from which only small quantities can be removed by washing with dilute alkali. That azine dyes likewise combine with filter-paper, various histological tissues, and no doubt numerous other substances is common knowledge.

However, no substances other than azine and azonium compounds (so far as is known at present) will combine in vivo, in visible form, with animal substances known to be actively proteolytic in nature, and of these only neutral red will combine in such a way that the physiological activity of the organism is unimpaired (Chlopin, '28). That other experimental substances than these dyes may so combine with enzymes as to partially and quantitatively inactive them, that many drugs may be enzyme adsorptive and produce the same effects of stimulation and depression as will be noted for the neutral-red reaction under various conditions is not doubted. But that these reactions are not made visible, that no details of cellular anatomy and physiology are thereby manifested puts them out of range for cytological consideration. Neutral red stands out as the most valuable reagent the cytologist has at hand for the investigation of cytoplasmic morphology and physiology.

From this standpoint, using a few unassailable phenomena such as the neutral-red reaction in the stomachs of various Protozoa and Metazoa as the bulk of the proof of a relationship between vital staining and active enzymes, the author ventures to extend the theory wherever the neutral-red reaction appears. The theory permits the extension by these additional phenomena with convincing elaboration, and also with the circumstantial confirmation of quantities of literature. That all of this evidence, considered in the light of enzyme reaction, is more or less suggestive and must be submitted to more definite proof is fully recognized. That it should, nevertheless, be presented here is necessary in order that the wide scope of the possibilities in careful and prolonged vital staining be adequately appreciated.

Notes

1. Shipley ('19), although he describes all phagocytosis or ingestion as similar to the process by which the Amoeba engulfs food, completely reverses all that he seemed to have aimed for and confuses the issue as follows: "In amoeba, whose cytoplasm is constantly and rapidly flowing in all directions, the food vacuole is passively moved about as the cytoplasm flows past. In the comparatively sluggish current of the protoplasm of the paramecium the food vacuole moves slowly over a route which exposes it at some time during its journey to the entire cell body. In the quiet cytoplasm of the cells of higher vertebrates, however, until movement is automatically mechanically checked by distention of the cytoplasm with masses of stored foreign material, the segregation granule is constantly and independently motile, and each one in its excursions covers a large part of the endoplasm of the cell. . . . The motility of the granule is an aid to the aggregation into its substance of particular matter which has passed through the cellular ectoplasm, since by this means the granule wanders over a large portion of the cytoplasm collecting foreign substance—combing the cytoplasm for food."

Why, then, are not his carmine, carbon particles, diatoms, bacteria, etc., found in the cytoplasm? Thus this altogether stimulating piece of work, by a strange twist of logic, loses all force and is now referred to only in the discussions of Lewis and Lewis ('24) and the Sabin School concerning preformed vacuoles. Such a conception involves the passage of substances through three membranes before assimilation could be possible. First, the cell membrane must receive foreign matter, sometimes useless or poisonous, and raw food materials directly into the living matrix of the cell; they must then pass through the vacuole membrane unchanged where, "in these tiny stomachs materials useful to the cell may be selected and separated from poisonous or useless matter," and where food materials may be acted upon by the various secretions of the cytoplasm poured into the vacuole; and again they must be passed through the vacuole membrane "to be reabsorbed as food by the cytoplasm or passed on to other cells for nourishment or to build up secretion" (quotations from Shipley). As for the waste materials, they must for the fourth time pass a membrane in being excreted? It is impossible to understand how such views can be reconciled with the very obvious behavior of Amoeba and Paramecium, especially in regard to the handling of large particles and experimental substances which Shipley himself describes so well.

Also, moving neutral-red bodies have never been seen in any normal metazoan cell studied in vivo. These cells may be assumed to be normal, as they are in organisms living and reproducing for long-continued periods. There is no independent motility in any neutral-red body and in attached tissue cells there is no motility at all. Leucocytes, to be sure, are always more or less amoeboid, but that the vacuoles move independently of the protoplasm is doubtful.

2. Evacuation in Paramecium is not the illuminating process that it is in Vorticella. It is extremely difficult to follow the order of the vacuoles, and in no case has it been possible to follow one vacuole from formation to evacuation when there are any considerable numbers present. When there are but very few only in the posterior end, the matter is not so difficult and individual vacuoles may be traced. The cytophyge in the three species of Paramecium studied, multimicronucleata, caudatum, and aurelia, is in approximately the same position—between the gullet and the posterior tip (Wenrich, '28). A vacuole at the cytophyge remains sometimes for a minute or two before emptying. It does not approach closely to the periphery of the wall, hindered, no doubt, by the parabasal bodies. Sometimes vacuoles slowly revolve for a time at this point, although more usually they are altogether quiet. Rarely two vacuoles coalesce. Then the vacuole opens into the short funnel of the cytophyge and the contents stream out through the narrow opening which is never observable except during the passage of visible particles. The vacuole retains its round shape during this process, never becoming spindle-shaped. The vacuole wall disappears in the walls of the cytophyge.

There is comparatively little here in the process of evacuation to favor the theory of a continuous system of vacuoles. There is no indication of a pulling force drawing the vacuoles up to the anus. No relationship is manifested between the vacuole just disappearing and the next to come. Save for the occasional coalescence of two vacuoles, the impression is decidedly one of discrete, entirely independent vacuoles, quite contrary to the suggestions at vacuole formation. Lewis and Lewis ('24) describe an experiment whereby the spherical neutral-red bodies of mesenchyme cells may be caused to become channel-like: "If a medium containing a dilute solution of urea is used on the culture and a minute fragment of urease tablet added, the vacuoles changes into channels which extend far out into the cytoplasm, as the urease breaks down the urea. If previously stained with neutral red the color disappears." Will such an experiment show continuity of the Paramecium vacuoles?

Solutions (0.025 per cent) of urea and urease tablet are added separately by means of a needle wet in the solutions to a hanging-drop preparation of perfectly stained Paramecium—animals from a continuously stained culture showing numerous and brilliant food vacuoles. Within two to three minutes, increased brilliancy in the vacuoles is noted, due to all of the vacuoles becoming the acid bright red. Then the mitochondria (see part on cellular synthetic system) become stained also with the decidedly acid reaction. (The urease solution is markedly alkaline with neutral red; also the urea solution, both giving yellow in the test tube.) (Neither reagent alone produces any visible effect upon the protozoan.)

Within the next five minutes, the food vacuoles lose color without going through a perceptible alkaline stage, the stained mitochondria become more numerous, locomotion is markedly slowed down, and the customary protoplasmic streaming is altered. Then it is noted that the food vacuoles are forming into groups. Two join, the vacuoles open and they flow together. It is not the sudden collision and merging of two bubbles; neither, it is true, is there any sign of a neck or a connecting canal. A third approaches this enlarged vacuole, remains perhaps a few seconds, and then joins in the same way. Vacuoles of this type, commonly made up of four or five individuals, are swept to the cytophyge. Their contents appear thoroughly mixed, but when the contents emerge, four or five separate little bundles or 'food balls' resolve themselves and are swept away by the cilia.

Four or five such compound vacuoles empty the organism completely of food vacuoles within ten minutes. The cilia of the gullet continue beating throughout the process, but new vacuoles are not formed. In the meantime, the mitochondria lose color and the organism shows no tinge of the dye. The crystals, which are normally grouped into anterior and posterior clumps, lose this arrangement and become evenly distributed throughout the cytoplasm. Each crystal appears in a separate vacuole. Mast ('26) recognizes this in the crystals of Amoeba proteus, but the condition has not been seen in Paramecium. The whole cytoplasm becomes highly vacuolated, so intensely vacuolated that no homogeneous groundwork is at all visible. Adherents of the alveolar theory of protoplasm would recognize a condition more ideal than the most vacuolated pictures of fixed protoplasm or of autolyzing cells (R. S. Lillie, '24). The crystals in their vacuoles become gradually more pale, although they do not disappear.

The animal revolves and makes some forward movement. Although almost obscured, the contractile vacuoles fill and empty regularly. If too great a quantity of the reagents has been added, these vacuoles increase enormously before death. In almost all experiments, however, death does not ensue. Within two hours, the cytoplasmic vacuoles imperceptibly diminish in size, a groundwork appears, the pale crystals clump posteriorly and anteriorly again, and the organism appears normal but still entirely devoid of food vacuoles. Within three hours, food-vacuole formation is fully reestablished and the newly formed vacuoles become perfectly and brilliantly stained. Nothing is added, the cultures are not rinsed nor aerated (they are kept sealed by adding water at the edges of the cover-slip), but the neutral-red reaction appears with the new food vacuoles as if stain had been freshly added to the medium.

One fact brought out by this remarkable performance is that the vacuoles of Paramecium may coalesce anywhere in the cytoplasm, this in a deliberate manner not suggestive of broken membranes. Whether or not this points to a continuity is a matter of interpretation; there is no definite proof at present.

A fact to be considered is the end products of the digestion of urea by urease, which are CO_2 and NH_4OH. Both of these substances have been shown by the Lewises ('24) and by Chambers ('28) to affect the vacuoles of cells markedly. The cell in this case returns to normalcy, showing no trace of the influence of either substance in the medium. The experimental production of complete evacuation of food from the organism, the initiation of a reversible condition of extreme vacuolization of a true cytoplasmic nature, the paling effect upon the crystals, etc., are all points of great interest, but the rationale is at present absolutely unknown.

The urea-urease reagents used in dilute solutions (1 cc. of 0.025 per cent solutions to 50 cc. of culture) upon cultures of stained Paramecium repeat the above phenomena and seem to have no injurious effect. An 80 per cent conjugation in P. multimicronucleata was noted in one culture eighteen hours after the addition of the reagents. The general effect produced in most cultures is the impression of unusual transparency of the cytoplasm following recovery. The unstained mitochondria and the brightly stained food vacuoles stand out in clear-cut relief.

Amoebae in the same culture do not recover. They make some attempt at evacuation, but it is not successful until the organism is injured beyond chance for recovery. This is suggestive of the higher efficiency of the continuous type of digestive system in which perhaps, there is a degree of peristalsis subject to some regulation.

3. No attempt was made to control temperature during staining. The temperature varied from 40° to 70°F. in the daytime.

4. A fact arguing against the existence of a continuous digestive system in ciliates is the reaction of vacuoles containing zoochlorellae. These vacuoles show no neutral-red staining; rather are they preserves for the symbionts. Yet once these zoochlorellae were ingested. What is now their relation to the digestive vacuoles?

THE CELLULAR SYNTHETIC SYSTEM

In neutral-red solutions of 1:15,000,000 down to 1:10,000,000 and more, only the food vacuoles in Paramecium stain. Beginning at about 1:5,000,000, another cellular constituent becomes definitely involved in the neutral-red reaction. The vacuoles stain first as described in the previous chapter; but within a short time there are noted a few red granules in the cytoplasm close to the vacuole membranes. These increase in number, especially in the region of the newly forming vacuole at the cytopharynx. They possess great and apparently independent activity. As the new vacuole is being formed they gather at the membrane, bombarding it like hailstones, but making no impression on the firm surface. Then as this vacuole flows aways attached by its canal, the outlines of which are, by the way, more sharply outlined by the border of red granules, some of the granules leave this vacuole and return to the next, which is already in the process of formation. Those left continue bombardment of the vacuole as the pink color slowly forms within. All of the vacuoles acquire numbers of these persistent satellites. Those at the newly forming vacuole are most numerous.

A number of red granules also appear, travelling about in the cytoplasm generally. These move at a much slower rate than those near the vacuoles. Usually they follow the protoplasmic streaming, but observation shows that within the stream there is considerable independent movement. A Paramecium at a concentration of neutral red greater than 1:5,000,000 is so highly stimulated that evacuation soon becomes rapid and within a short period there are no vacuoles in the anterior end of the organism. There are then comparatively few granules in the anterior end, while they are numerous around the few posterior vacuoles and in the cytoplasm, giving the posterior region much more brilliancy than the anterior.

Paramecium remains in this condition for some time. Vacuole formation is at a steady, rather slow rate and defecation at the same rate keeps the food vacuoles limited so that they are not pushed to the anterior end. Locomotion or contractile-vacuole activities are in no way interfered with. After about twenty hours to forty hours, the red granules disappear gradually, more food vacuoles are formed and pushed to the anterior of the organism, and by the third day the animals appear as normal as those which have been stained in greater dilutions. They differ in no manner from control cultures, save that all the food vacuoles are brightly stained. In this condition they may remain stained for seven or eight days.

A 1:1,000,000 dilution is not favorable for Paramecium. They may live for two or three days, the red granules becoming more numerous and the food vacuoles being diminished to two or three watery-looking vesicles which are emptied so rapidly that they sometimes do not stain at all, although they are so surrounded with red granules as to seem at first glance to be well stained. Locomotion becomes slow, the anterior end, which contains only red granules and crystals, becomes somewhat atrophied and sharply pointed, with sometimes an abortive effort to pinch off. There is a great deal of resemblance in this condition to Calkins' ('09) picture of protoplasmic stability where "reserve food granules fill the protoplasm, there are no food vacuoles, food taking and movement stop, dividing stops and the animal unless treated invariably dies." It is a picture of exhaustion following hyperstimulation. P. aurelia often survives this condition; P. caudatum and multimicronucleata practically never after forty-eight hours.

At dilutions less than 1:1,000,000 another set of granules is involved in the staining. These are definitely rod-shaped, immobile bodies underneath the pellicle. They make out the ciliary pattern so brilliantly that it is clear that there is one granule for each cilium—these granules are the ciliary parabasal apparatus. No Paramecia have ever been noted to survive staining of the bodies.

In Stentor some of the cytoplasmic granules are always stained in the weakest dilutions, while one as strong as 1:5,000,000 will bring out the ciliary pattern with delicate precision, these parabasal bodies, however, being granules of the same size and shape as those of the interior cytoplasm. Those in the general cytoplasm move about with deliberation; at a rough estimate approximately 20 per cent of them are in the diplococci form. They are not excessively numerous about the food vacuoles, so that distribution is quite even throughout the organism. Such staining is not toxic, as one rich culture was kept constantly stained in this condition from December 18th to March 20th.

In Vorticella, as in Paramecium, the red granules are intimately associated with the food vacuoles clustering closely about them. Others in the cytoplasm move freely, whereas others underneath the pellicle are arranged in definite horizontal rows. There are no cilia below the oral groove in this form; nevertheless, the arrangement is altogether like that of the parabasal bodies in Paramecium and in Stentor, except that in these forms the rows are vertical. Vorticella seldom survives such complete staining. A hanging-drop preparation showed, after eighteen hours, many of the parabasal granules merged in great clumps just underneath the pellicle, and especially on the disk they formed projections.

Histrio, typical of hypotrichs in staining reactions, shows very few granules, only in the cytoplasm, in a 1:1,000,000 solution. These are for the most part immediately underneath the pellicle. There are also a few, large and quite stationary, adherent to the one large vacuole at the base of the cytopharynx.

The size, movement, and orientation of these cytoplasmic staining bodies in various ciliates recall the series of papers by Horning in 1928 on the mitochondria in ciliates and Amoeba. Causey's work in 1926 on Paramecium also definitely establishes these bodies as mitochondria, readily demonstrated by mitochondrial fixing and staining methods. They also respond to Janus green. Causey shows that the parabasal bodies are a 'kind of mitochondria' or at least a derivative of mitochondria, differing from them only as to being insoluble in acetic acid. Fauré-Fremiet, in 1910, described the neutral-red staining of mitochondria in Protozoa. Nath, in 1928, stained mitochondria in spider eggs with neutral red.

How does the staining of mitochondria with an azine dye fit in with the conception that such a reaction shows enzyme relationship? Could the enzyme be hydrolytic here, for there is no vacuole and consequent separation and protection from the cell matrix? Instead, there is the most intimate contact with the cytoplasm and the nucleus.

The explanation is at hand, suggested by the researches of the great physiologist who did so much with enzymes, Bayliss, ('23):

It is sufficient to recall the fact that the equilibrium position in a reversible hydrolytic reaction depends on the concentration of water in the system to see that the same enzyme will have either hydrolytic or synthetic action according to circumstances. There is no necessity for the assumption of special synthesizing enzymes, and indeed, an examination which I made of the cases in which they were supposed to exist showed the falsity of the hypothesis. We see therefore that what is wanted to control the sign of the activity of enzymes in the living cell is an effective method of changing the concentration of free water.

If in the watery vacuoles of the cell the enzymes are hydrolytic, in the cell matrix they are synthetic. This is suggestive that in the cytoplasm enzymes of the same nature that are poured into the cell vacuoles are borne upon the mitochondria and that upon these lipoidal surfaces such enzymes are synthetic. Marston ('23) recognizes this possibility: "The mitochondria may be thus the site of synthesis in the cell, the water-poor phases which exist at the surface of the lipoid constituents of the mitochondria instituting favorable conditions for the synthetic activities of the enzymes."

A hypothesis distinguishing between cellular hydrolysis and synthesis gives fair promise of clearing up many difficulties. There is little enough in the literature concerning the hydrolytic properties of cells. A vague specificity for certain food is known to exist—Amoeba will eat many things that Paramecia will not; mononuclears will ingest substances that polynuclears pass by; nerve cells certainly have no opportunity to ingest materials which are presented to the Kupffer cells nor do they possess the apparatus for so doing. But no matter how carefully food is selected from the medium, or prepared by organismal hormones, it is still foreign substance to the cell—foreign protein elements, fats, and carbohydrates. It is well recognized that from these materials each cell synthesizes its own native protein, etc.; but some degree of hydrolysis must take place before materials for synthesis are available. In every cell, therefore, the cytologist might seek some type of hydrolyzing apparatus—this a vacuolar system.

On the other hand, the cytological literature is so full of conflicting explanations of synthesizing processes that the confusion is overwhelming. (See bibliographies of Cowdry, '24; Bowen, '26; Gatenby and Nath, '26; Nath, '26; Harvey, '27; Gardiner, '27; Hibbard, '28; Kater, '28.) None of these papers expresses appreciation of a preliminary hydrolytic process and it may be the confusion of these two processes and their structures (see part on the morphology of the neutral-red bodies and their relation to the Golgi and mitochondria) which prevents the total lack of satisfactory conclusions. The literature on yolk formation alone demonstrates the situation; the student of the subject feels as if he might as well draw lots to determine which side he should take in considering the problem of whether it is the Golgi bodies or the mitochondria which form yolk. A new approach to the problem may have a clearing effect.

Nath ('26) concludes: "In the present state of our knowledge it is not possible to make any general statement with regard to the functions either of the mitochondria or the Golgi apparatus." It seems decidedly worth while to consider, however, that the mitochondria, as bearers of synthetic enzymes, are in all probability the immediate agents of cellular elaborations and secretions, but that the source of the materials with which they work is the product of the hydrolytic system of the cell.

The two processes, hydrolysis and synthesis, are not to be confounded and visible structures revealing them are to be studied with the physiological viewpoint in mind.

Notes

1. By no means may all mitochondria be stained by neutral red without injuring the cell. Presumably the process of staining is as follows: Neutral red, in inactivating a given amount of vacuolar enzyme, stimulates the secretion of more enzyme. Increased hydrolytic enzyme production slightly injures the vacuole membrane, causing it to become permeable to neutral red. Admitted into the cytoplasm, the stain is adsorbed by the synthetic enzymes on the surfaces of the mitochondria. Paramecia live, divide, and conjugate with both vacuoles and mitochondria stained, provided there be not too great stimulation of enzyme secretion. Among metazoan cells, however, ready permeability of the vacuole walls to neutral red is rare.

2. Amoeba limax. Amoeba limax cannot be stained in any dilution used, from 1: 1,000,000 to 1: 15,000,000, without staining the mitochondria. During the first twelve hours or so, the amoeba shows only stained food vacuoles; these are small, solid-appearing, and quite bright in color. But after the first day, the vacuoles disappear and only mitochondria are seen. They are granular and exceedingly numerous; they do not stain red, but a brilliant reddish purple so that, small as this amoeba is, it is easily distinguished under low power as a flowing organism filled with uniformly stained purple granules. Such a condition does not seem to be harmful, as it persists in all dilutions over 1: 2,000,000 for seven or eight days, the granules becoming more pale.

Color changes in the dye Janus green B during the staining of mitochondria are described by Cowdry ('24) and by Marston ('23), the intense blue of the dye being reduced to red and then to a colorless condition. No such color changes are described for neutral red anywhere in the literature. Becher, in 1926, used it in staining Opalina and Paramecium and found that it could not be reduced in the hydrogen chamber. Fauré-Fremiet's ('10) work on neutral red is suggestive, however: neutral fats and their solvents in a solution of neutral red form the yellow base of dimethyldiaminotoluazine upon the surface of the fat-droplets and, this yellow base being insoluble in water and readily soluble in fat, the droplets are stained vivid yellows and orange. A fatty acid forms a red soap; monobasic acids form a bright red color; di- and tribasic acids give blue and green colors.

In all the ciliates examined, both in the food vacuoles and the mitochondria, color reactions are quite within the usual range of neutral red as an indicator and they always remain so until the dye is eliminated from the organisms. In limax, however, and some other forms, the nature of the dye must either be changed in passing through the walls of the food vacuoles or by the nature of the mitochondria. Since this change is not pathological, as in the case of the reactions in Janus-green staining, it will probably be of value to determine its significance.

3. Amoeba radiosa. Amoeba radiosa is much like limax in that the mitochondria are peculiarly susceptible to neutral red. The comparatively few, small food vacuoles are not seen after the first twenty-four hours and the mitochondria are very numerous. As in A. limax, they are small and granular, but they do not stain with quite the purple intensity of limax. The color is more of a magenta red. Although the mitochondria are very active, they do not extend far out into the pseudopodia, progressing only a little distance, then returning, as in Strangeway's ('27) tissue-culture cells.

4. Amoeba polypodia. This form closely resembles A. radiosa, the mitochondria staining vividly a magenta color.

5. Amoeba proteus. In neutral-red dilutions of from 1: 15,000,000 down to 1: 2,000,000 Amoeba proteus shows no staining of the mitochondria. There are three types of essential cytoplasmic inclusions (Schaeffer, '16), the crystals, and the alpha and beta granules. In the 1: 2,000,000 stain there is, after about eighteen hours, a scarcely perceptible pink refringency about all of these structures which increases so little within the next few days that it is a question whether this is staining or some light effect. With a 1: 1,000,000 stain it is definite that each of these structures is subject to the neutral-red reaction.

The alpha and beta granules are much larger than the mitochondria in Paramecium. They are never solidly stained; it seems that staining is a surface reaction of such delicate nature that the refringent halo is very narrow. After twenty-four to thirty-six hours, the crystals, on the other hand, show a considerable area of reddish brown to orange staining about each one. No limiting membrane can be observed: however, Mast ('26) says: "Each crystal is in a vacuole. The vacuoles contain a saturated solution of the substances of which the crystals are composed." The presence of the vacuole is entirely confirmed by the urea-urease reaction. (See part on cellular hydrolytic system.)

Three types of cytoplasmic inclusions (not considering food vacuoles) stain in Amoeba proteus; do they thus give evidence of enzyme action upon their surfaces? Which are mitochondria, or is each a kind of mitochondria? Causey ('26), in describing the nature of the parabasal bodies in Paramecium as very closely related to the mitochondria, recognizes the possibility of there being more than one kind: "The first step in advance will probably be the development of a technique which will differentiate between kinds of mitochondria, in which morphology must play a minor or purely physical part."

The food vacuoles in A. proteus do not disappear even with heavy staining of the cytoplasmic bodies. Stimulation is revealed by numerous cell divisions, so that this form, more than any other protozoan studied, increases rapidly in stained cultures. The same phenomenon, to a much greater extent, was observed with Stenostomum, a flat worm which reproduces by budding. These exceedingly small worms stain very brilliantly, due to the staining of the rhabdites of the ectoderm (see part on elective staining) and, increasing in great numbers, they made the sides of the dishes and top of the water red. The control dishes showed quite moderate numbers. Rhabdites may be of mitochondrial origin.

(If there are different 'kinds of mitochondria' to be distinguished, there seems to be some basis for predicting that one of these will be concerned with cellular respiration. Joyet-Lavergne ('27) has indicated that glutathione may be held by the chondriome.)

6. Amoeba verrucosa. A. verrucosa seems to thrive equally well in all dilutions from 1: 1,000,000 to 1: 15,000,000, retaining the stain on an average of about a week. Only in the 1: 1,000,000 are there any stained cytoplasmic granules. In A. proteus none of the stained granules are in any way associated with the food vacuoles, but are uniformly distributed throughout the cytoplasm. In A. verrucosa, however, there is a clustering, especially about newly formed vacuoles. The phenomenon is never prominent, because there are very few granules and they gather only on one side of the vacuole, forming a cap. They are always a bright red and appear to be solidly stained as in Paramecium.

7. Actinophrys sol and Actinospherium. These organisms are especially interesting because of the mitochondria, which stain a deep purple in each form. Actinophrys has rod-shaped bodies which travel in a definite regular row out to the very tip of each slender pseudopod, turn and travel back in another line so that each pseudopod has a continuous two-way procession. Actinospherium has granular mitochondria, and while they also proceed to the tips of the pseudopods, there are no definite lines. In Actinospherium there is no definite orientation of the mitochondria just beneath the membrane of the body proper, although they are numerous in this region; while in Actinophrys there is a single

row, closely spaced, with the mitochondria lying parallel to the membrane. These seem to be almost stationary.

The *food vacuoles in each form* are densely clustered in the center of the organism. Individual vacuoles are most often very small, as one would expect from the size of the ray-like pseudopods which draw food particles (in this case small particles only) into the body and so invaginate to form the vacuoles. These are to be contrasted with the enormous vacuoles of A. proteus, the rather large ones of Arcella vulgaris, etc.

8. Heterophrys myriopoda and Clathrulina elegans. In these forms no mitochondria stain even in a 1: 1,000,000 dilution. Food vacuoles form a central mass of very small, bright bodies.

9. Amoeba guttula and Amoeba chaos. After several hours in neutral-red solutions, the mitochondria in these forms, naturally invisible, become distinct. They are not stained, seemingly, but they appear dark and somewhat refringent. They may soon be lost sight of, however, in clustering about the darkly stained outer walls of the food vacuoles.

10. Causey's account of the behavior of the mitochondria in Paramecium stained with Janus green accords for the most part quite accurately with their behavior stained in neutral red. His statement, "The first indication of staining is in the region at the end of the cytopharynx where presumably the stain is first absorbed," favors the idea of the vacuole membrane as the site of permeability to colloidal matter.

Exception must be taken to the following statement, however: "There can be no question regarding the lack of independent motility on the part of the mitochondria, as in the vitally stained organism they can be followed through cyclosis passively accompanying the food vacuoles and never showing independent motility." Horning ('28) found just the opposite in an amoeba, the mitochondria bombarding the food vacuoles; and such is certainly the case in the Paramecia studied. Especially at the base of the cytopharynx and around the newly formed vacuole is violent movement observed. And there is always some movement among the mitochondria accompanying old vacuoles. As for the slow-moving mitochondria of the cytoplasm, their independence of protoplasmic streaming is obvious, especially during conjugation.

The writer has never found any food vacuoles present during conjugation in P. multimicronucleata nor in P. caudatum. Landis ('25) makes no reference to the absence of food vacuoles and Causey ('26) states that they are reduced, the extent of reduction depending upon the feeding activities of the organism before conjugation. Consequently, he says, the number of mitochondria is reduced, "the mitochondria present depending upon the number of food vacuoles present and their contents." Now in the conjugants observed from a continuously stained culture of P. multimicronucleata with no food vacuoles present in the conjugants, the stained mitochondria are very numerous. They are more evenly distributed throughout the cytoplasm than is ever the case when food vacuoles are present and, as a whole, they more nearly conform to the general protoplasmic streaming. In fact, the steady flow of the whole cytoplasm about the large, clear space occupied by the nuclei is so rhythmical as to be in striking contrast to the more heterogeneous movement in feeding individuals.

The non-conjugating individuals at this time are full of food vacuoles and have few or no stained mitochondria (the stain being of very great dilution), yet the conjugants show the cytoplasm thickly studded with stained mitochondria, thereby producing in the conjugants more brilliancy than in the separate individuals. One is prone to conclude that synthetic processes are intense, occasioned by nuclear and mitochondrial exchanges.

If a 1: 5,000,000 stain be added to an unstained culture in which there is conjugation, the non-conjugating individuals show stain only in the food vacuoles, none on the mitochondria. The conjugants, however, with no vacuoles quickly show staining of numerous mitochondria. If it can be proved, as Landis ('25) states, that the mouth is obliterated during conjugation and that there is no entrance through even a very abbreviated invagination, but only through the pellicle, it would be a good argument against the theories developed herein as to the reception of colloidal particles in cells. The writer cannot believe this to be the case, however. Ciliary action in the region of the gullet is obvious, just as it is throughout the urea-urease reaction (see part on hydrolytic system), but the type of peristalsis (?), the contractions of which form vacuoles, is inhibited. Such inhibition occurs during hyperstimulation after initial staining, and during the urea-urease reaction. The hunger stimulants are not present.

Food vacuoles are present during division in Paramecium and stained mitochondria are not noted when the non-dividing organisms show none. Metabolic conditions are obviously much different from those existing during conjugation. The writer has not found any clues as to the manner in which the new digestive system is formed in the daughter cells.

Diplococci forms of mitochondria appear and disappear rapidly in Paramecium. These forms are always present and there is no period at which they seem to be more in abundance than at other times, unless it be the period of the rapid appearance of new mitochondria during initial staining in high concentrations of the dye (around 1: 2,000,000). At this time, however, division is too rapid for accurate observation. Nevertheless, the author cannot support Causey's statement: "No evidence for the division of the mitochondria at any time is found; they appear, instead, to arise de novo." It is true that Paramecium is no such striking example for mitochondrial division as is Stentor. Also the rush of numerous mitochondria to the base of the cytopharynx is remarkable, but in a cell with the focusing depth of Paramecium this may not be enough grounds for stating that it is in this region that the mitochondria arise de novo.

INTRA- AND EXTRACELLULAR DIGESTION

Certain animals with a digestive lumen are known to have only intracellular digestion. Metschnikoff ('05) showed how actinians, Planaria, and leeches take food into the digestive cavity, where it is seized by the lining amoeboid cells and digested by them individually. Surplus food may be ejected from the lumen with no sign of enzyme action having taken place upon it.

Willier, Hyman, and Rifenburgh, in 1925, made a histochemical study of digestion in Planaria and conclude: "As soon as the food enters the intestine the phagocytic cells begin to engulf it in an amoeboid manner, forming typical food vacuoles."

Such facts are quickly and completely demonstrated by the neutral-red reaction. The intestinal cells become heavily stained with neutral red in Planaria, which have densely packed neutral-red bodies; there are fewer food vacuoles in the large, clear endodermal cells of Hydra. The digestive lumen in neither of these forms ever shows a trace of neutral-red staining.

All rotifers so far examined are excellent examples of purely intracellular staining. In dilutions of the stain from 1:1,000,000 to 1:15,000,000 the cells of the stomach or intestine stain brilliantly. In Brachionus urceolaris it is not difficult to see that the digestive cells are ciliated and also capable of amoeboid changes in shape. The food vacuoles are large and closely packed. There are some slightly refractive granules which do not stain and are probably secretion products.

The rotifer ingests small particles, or at least they are small by the time they pass the mastix. For a time they may be seen in the stomach lumen whirled about by the long cilia, and then they disappear. Of course, the actual process of engulfing is not visible, so great is the tangle of swirling cilia. No trace of stain is ever noted in the lumen of the entire digestive tract.

A different picture is presented by Chaetogaster langi, a small, very transparent oligochaete worm. Within ten to fifteen minutes after staining in a 1:1,000,000 or 1:5,000,000 dilution, the first indication of the dye is a clear, bright pink color in the lumen of the crop. The crop, posterior to the pharynx which never stains, is a comparatively long organ lined by small cells which do not take any stain at this time. Within another ten or fifteen minutes, faintly colored vacuoles appear in the cells of the gizzard, which is separated from the crop by a sphincter. These gizzard cells are large, ciliated, and somewhat amoeboid and soon become brilliantly stained, the vacuoles brick red shading to orange. Behind the crop is a straight intestine, widening slightly in regions where new buds are forming, no part of the lumen nor cells of which show any trace of staining. (See part on elective staining and secretion.)

Arcella seem to be the preferred food and but few are ingested at a time, so that no process of the gross digestive phenomena is obscured. The lumen of the crop is a bright pink, but the color of the Arcella, provided it be not a too old brown specimen which is rarely taken in, becomes a more intense red color. Much of the dye is evidently concentrated upon the surface of the shell and no doubt there is more in the interior. (To emphasize, none of the cells of the crop epithelium show any neutral-red staining.)

This is certainly not a cellular reaction, yet the picture is altogether similar to that found constantly in Amoeba proteus, which ingests Arcella. There the Arcella is suspended in a vacuole the narrow lumen of which becomes pink, and the longer the Arcella remains in the vacuole the more intense becomes the color upon its surface. In the worm the differences are that the lumen is already pink when the Arcella is admitted and it is somewhat larger so that more than one Arcella may be accommodated at a time. Digestion is therefore quickly initiated and more serviceable.

After a time a peristaltic wave expels the brilliant red Arcella into the lumen of the gizzard. Instantly all color on and in the shell is lost—neither the Arcella nor the lumen of the gizzard shows a trace of color. Yet that some fluid passes with the shell through the sphincter is certain.

In the gizzard the now colorless Arcella is continually revolved about by the action of the long cilia of the brilliantly colored epithelial cells. After a period averaging half an hour, the Arcella, usually looking somewhat crumpled if it be a particularly young specimen, is expelled into the intestine, and so into the rectum.

Digestion then in this worm is as follows: The cells of the crop secrete into the lumen an enzyme, or enzymes. Such enzymes are precipitated by neutral red, and the medium being acid, the result is a clear, solid core of pink color. Since enzymes are concentrated at the surfaces upon which they act as catalysts, neutral red in the compound demonstrates this concentration upon the food material in intensified color. This is a picture of true extracellular digestion, the lumen being surrounded not by a vacuole wall, but by a lining of cells.

The Arcella then passes a sphincter into a medium where there are not only no enzymes, but those passing with the shell are immediately inactivated by something in the gizzard lumen, thus releasing the neutral red from combination and rendering it invisible. (Marston's work and also Epstein's make clear that such a release is quite possible.) The gizzard cells are thus protected and digestion becomes altogether intracellular. (There is no indication of the pH of the gizzard-lumen fluid.)

The intestine shows no apparatus for further digestion. There is neither a secretion into the lumen made visible, nor cells with neutral-red bodies sufficient in size and number for more than their own individual requirements for nutrition. The intestine is plainly absorptive in function.

However, the cells of the gizzard have absorbed into their own bodies the greater part of the substance of the food. What is left for the intestine to absorb? Plainly very little. The problem is settled by a digestive phenomenon of great interest. Between five and ten minutes after an Arcella leaves the gizzard, two or three of the cells of the gizzard are expelled, following the Arcella into the intestine. More cells follow, extruded at intervals. The greatest number noted between one group of Arcella and the next was twelve. These cells are unmistakably of the gizzard epithelium—large, no other cells of the body approaching them in size, and so characteristic with their numerous stained granules and especially long cilia that they cannot be confused with any other cells nor with any foreign food material. In the intestine they are revolved about by the intestinal lining cilia and by their own active cilia, but by the time they have reached the rectum the neutral-red bodies lose color, the cytoplasm becomes faintly stained and then goes into solution, losing all color and becoming invisible. The granule-like neutral-red bodies do not disintegrate, but are excreted as pale yellow to colorless bodies. (This process is not the result of injurious staining effects, as it was later seen in unstained animals.)

The most reasonable explanation is as follows: Gizzard cells finish digestion begun by the free enzymes of the crop by absorbing the liquefied Arcella contents, further hydrolyzing them in the numerous neutral-red bodies and probably admitting the soluble nutriment into their cytoplasm. But an apparatus for releasing the prepared food substance into the body cavity seems to be lacking, and, instead, fully loaded cells are released into the intestine, where autocytolysis sets free the food material for the intestinal cells to absorb. It is interesting that the colorless food vacuoles, no doubt containing waste materials, are excreted intact. There are numerous examples of cells like the goblet cells being destroyed in the process of secretion and this may be a primitive modification of such a function.

Chaetogaster stands as a perfect example of the combination of the two types of digestion, each taking place in a definite region of the alimentary canal of the organism. Extracellular digestion is manifested by the neutral-red reaction, the whole lumen being stained as a solid core of bright pink. The lining cells have nothing to do with digestion after giving up their secretions into the lumen. The actual digestive processes are as removed from cellular participation as they would be in a test-tube provided with enzymes. (For a discussion of the neutral-red reaction of these cells, see part on elective staining and secretion.)

Intracellular digestion takes place in a region of the alimentary tract containing no enzymes in the lumen and inactivating whatever enzymes may be carried over into it.

Instead, the lining cells, by means of cilia and pseudopods, take into themselves, by way of amoeba-like food vacuoles, substances which they digest in these vacuoles, or lumina.

In spite of the striking dissimilarity between the pictures presented by extra- and intracellular digestive reactions to neutral red, the processes are essentially identical. In both cases enzymes, existing in a non-hydrolytic state in the cytoplasm, are released into watery lumina where they become hydrolytically active. Neutral red seems to indicate enzyme action wherever such action may take place.

Daphnia provides a perfect picture of the solely extracellular digestive activities of the alimentary tract. In a 1:1,000,000 solution of neutral red the canal curves through the body like a pink ribbon. When more dilute stain is used, 1:2,000,000 and over, only the anterior part of the tract is definitely pink (always excepting the short oesophagus), the color fading off toward the middle and posterior region. There are no sphincters and no inactivating substances in the tract. It is probably the anterior region that secretes the greater amount of enzyme, but these cells may be stimulated to secrete greater amounts so that the secretions extend far back in the lumen, or the posterior region may also be stimulated to secretion. It must not be forgotten that neutral red partially inactivates enzymes and thereby stimulates, perhaps, further enzyme production. The truest pictures of normal conditions can then be obtained only by using the weakest dilutions which will stain the organism completely. Thus a strong solution will mask the fact that the first half of the digestive tract is largely secretive, while the last half is mainly absorptive. No digestive cells are affected by the staining of the lumen—none exhibit the slightest neutral-red reaction in initial staining. (See part on elective staining and secretion.)

Résumé

In the digestive lumen of Coelenterates and Platyhelminthes where it has been repeatedly shown that digestion does not take place and that no digestive fluids are present, there no neutral-red staining occurs. In phagocytic amoeboid cells where it is known that digestion does take place, staining in the form of large and numerous neutral-red bodies is demonstrated. In forms showing no amoeboid cells in the digestive system and in which it is obvious that there is extracellular digestion, such a process is manifested by the neutral-red staining of the fluid of the lumen.

A mechanism for the immediate inactivation of free extracellular enzymes introduced into a region of amoeboid cells shows an 'anti-enzyme' effect made clear by a striking color reaction. One rôle of a sphincter between types of digestion is illustrated.

No example of intra- and extracellular digestion existing in the same portion of the digestive tract has been noted.

Notes

1. Chaetogaster limniae responds to neutral red exactly as does C. langi. Aeolosoma quaternarium, closely related to these worms, will not stain at all, even in a 1: 1,000,000. At lower dilutions it shows lumen staining throughout the tract, except for rather long stomatodeal and proctodeal regions, which do not stain. Two other small oligochaete species show only intracellular digestion.

2. Some small, free-living nematodes encountered in protozoan cultures have extracellular digestion, as indicated by a slender line of bright red down the center of the lumen. The digestive cells are so darkly pigmented that it cannot be made out whether they show any intracellular digestive functions.

3. Gastrotrichs have only intracellular digestion.

4. The digestive tract need not be acid in reaction; the aquatic larva of a midge (not identified) shows a uniformly alkaline lumen—a clear yellow—and has only extracellular digestion.

5. Janus green B is thought to be specific for mitochondria (Cowdry, '24), although some few workers have mentioned its transient appearance in the cell vacuoles before the mitochondria are stained. As an azine dye, however, it should demonstrate enzymes in both the hydrolytic and synthetic states. In Daphnia, starting with dilutions similar to those used for neutral red, it is immediately apparent that freshly prepared Janus green stains the contents of the lumen in exactly the same manner as does neutral red, save that the dilutions are manifestly too strong. Dilutions of 1: 2,000,000 up to 1: 10,000,000 quickly stain the whole tract a vivid green, and within a few hours the animals die with violent constrictions of the intestine. In a 1: 20,000,000 dilution the staining is much less intense and the animals live for twelve hours, their embryos being hatched and surviving for several hours. At 1: 50,000,000 staining of the lumen appears quite normal—within half an hour after staining, the anterior half of

the digestive tract only is a light, clear green, fading off in the posterior region. These animals will live for twenty-four hours and their young for a week or more, but they will not mature.

In a 1: 100,000,000 solution staining is likewise apparent within half an hour; the adults survive for a little more than forty-eight hours and the young are numerous, but fail to develop to maturity. Janus green therefore is not specific for synthetic enzymes only (mitochondrial staining), but presumably it is so toxic, especially in the dilutions that have been used by cytologists, that it injures the cell vacuolar membranes, almost instantaneously rendering them very permeable, so that the dye passes into the cytoplasm and attacks the mitochondria until in about ten minutes (Cowdry, '24) they become invisible and the cell dies. (See discussion of Popa's work with Janus green in part on the development of the sperm and fertilization.) Fresh safranin is even more toxic to Daphnia; however, a 1: 100,000,000 solution demonstrates the lumen enzymes perfectly. Old solutions lose somewhat in toxicity.

ELECTIVE STAINING AND SECRETION

Metschnikoff ('05), in working with anemones and Planaria, describes only the staining of the digestive systems of these animals. However, staining in these forms does not stop with the digestive system.

Hydra vulgaris (pale yellow variety) shows complete staining of the endoderm within a short time after remaining in a solution of 1: 5,000,000 neutral red or after a meal consisting of a well-stained Daphnia. This includes the layer of endodermal tissue extending to the tip of each tentacle. Within at least twenty-four hours after this initial staining, mesodermal elements show a sparse scattering of extremely small neutral-red bodies, and ectodermal cells have small, brilliant neutral-red bodies definitely oriented with respect to the nematocysts.

Microhydra hydrandths show the same staining reactions and retain the stain, especially in the endoderm, for long periods—eight days in a 1: 2,000,000 solution. No medusae have been seen.

Three Turbellaria have been studied. Planaria foremanii and a very small planarian stain alike—the intestinal cells first and always most prominently, and later scattered cells in the ectoderm.

A third form, however, Stenostomum tenuicauda, varies the order of staining found thus far in coelenterates and flatworms. The ectoderm stains first, responding almost instantly to any dilution of neutral red from 1: 1,000,000 to 1: 10,000,000 and also staining in such weak concentrations that no Protozoa, even the delicately susceptible Stentor and Paramecium, respond. It is the rhabdites of the ciliated epidermal cells that stain so avidly, and they take a dense, deep red color unlike any of the ordinary colors in the neutral-red range. The rhabdites are in closely packed groups in each cell, oriented as the parabasal bodies in ciliated Protozoa are. Why do such structures stain with neutral red? Their relation to enzymes at least is suggested.

The intestinal cells stain as in other flatworms, although they are always obscured by the ectodermal staining. They also lose their stain at about the same time as does Amoeba proteus in the cultures. The worm, however, remains brilliantly stained for from two to three weeks, due to the densely colored rhabdites.

Two rotifers have been selected for study as being typical of staining reactions among this group. Brachionus urceolaris represents the majority of forms encountered. A 1: 1,000,000 dilution of neutral red is a favorable medium, although rotifers seem not to suffer in slightly more concentrated solutions. After an hour in this dilution, the cells of the stomach have become heavily stained. These are large, ciliated cells with numerous neutral-red bodies of acid reaction for the most part, although each cell seems to have a few ranging into the alkaline shades. The lumen of the digestive tract does not stain.

After two hours approximately, two more types of cells have stained almost simultaneously. First, on either side of the stomach in this form are branching organs, the hepatic caeca. The cells are large, with distinct nuclei; the neutral-red bodies are arranged in a rather wide circumnuclear grouping and show a light, bright acid reaction. Although by no means all of these red bodies are visibly connected with each other, they give the impression of being a continuous system and not discrete bodies. Secondly, the ovaries are stained, provided they contain young eggs.

For approximately three hours, the animals remain with these three distinct regions stained. Then other systems gradually take up the neutral red until every cell in the organism shows neutral-red bodies within five hours. These bodies are so distinctive for the various types of cells that they bring out a histological map of the rotifer that is well worth detailed attention. No staining of the coelomic fluid or digestive-tract lumen occurs at any time. All staining is intracellular, and staining proceeds in definite order.

In the second form, Philidina (transparent and colorless), the progress of staining is similar. The cells of the internal organs are soon obscured, however, by the distinctive staining of the epidermis. This structure from the neck region to the tail is a pattern of delicate rod-shaped bodies which stain a rosy heliotrope. The individual cells are difficult to make out, but it is judged that these bodies are regularly arranged in each cell, producing a general uniformity over the whole animal that recalls the perfect ciliary pattern brought out by the parabasal bodies in Stentor and in Paramecium and also the rhabdites of Stenostomum. This epidermal staining persists long after the neutral-red bodies of all other organs have disappeared.

Daphnia is a most favorable form in which to show the progress of a definite course of staining in an organism. A 1:2,000,000 solution is an ideal medium. Within from fifteen minutes to an hour, the lumen of the digestive tract just behind the oesophagus shows a clear, light pink. Soon the lumina of the hepatic caeca are also pink. This reaction extends to the midgut and fades off posteriorly. During this period no cells of the digestive tract show any trace of neutral red.

For about two hours, the animal remains with only this bright pink core showing the presence of neutral red. Then it is noted that an extensive system of large cells connected by long processes is beginning to show neutral-red bodies. These cells lie loosely about the whole length of the digestive tract, some about the heart, some in the respiratory appendages, and a few in the brain region, together forming a great anastomosing group known as the phagocytic system (for latest reference, see Goodrich, '28). The vacuoles in these cells rapidly become brilliant, the neutral brick red predominating, so that the whole system is picked out in contrast to the only other stained portion of the animal, the pink lumen of the foregut.

It is especially to be considered that the cells of the digestive tract at this time show no trace of neutral red. These cells are very small in contrast to the great phagocytic cells, but it can be ascertained beyond doubt that they show no staining. It is generally assumed that there is no passage of material from the alimentary tract into the body except by way of the digestive epithelium, "the barrier between the living tissue and the lumen contents" (Macklin and Macklin, '28). The digestive cells, therefore, in Daphnia are permeable to neutral red, yet the stain passes through them invisibly. There is no 'neutral-red reaction.'

As the phagocytic cells are being stained, a second, much less noticeable system begins to show neutral-red bodies. These cells are somewhat smaller, fewer, and more loosely connected than the phagocytic cells, but they are similar in distribution and in manner of connection. Their neutral-red bodies never stand out brilliantly, as they are usually on the alkaline side of the dye and the pale orange and yellow coloration of the bodies is best noted under the higher powers of the microscope. These cells are neglected by Daphnia morphologists, so they may be given the name of the 'alkaline phagocytes.'

Almost contemporaneous with the staining of these cells, the gonads begin to show brilliant spots of color. It is the younger end of the egg string only which shows any neutral-red bodies in the germ cells (this is the posterior end of the ovary; Claus, '76), while it is the older, anterior end which has supporting cells with enormous vacuoles so that the neutral red in them may produce great masses of color. Developing eggs with any considerable amount of yolk show no staining. In males (rarely noted in the cultures) none of the germ cells stain, although the small supporting cells of the testis become brilliant at this stage, corresponding to the staining of the ovaries in the female.

It may be assumed with some degree of reason that these three systems of cellular elements—the neutral and alkaline phagocytic systems and the gonads—are the first to receive substances coming from the digestive tract. When the dilution of the dye is greater than 1: 5,000,000, no other cells than these become stained, although these elements may retain the stain for forty-eight hours and longer. Now in Daphnia there is no closed circulatory system. All the cells alike are bathed in the body fluid; all the cells have practically equal opportunities for taking up substances from the medium; substances in the body fluid are kept in rapid circulation by the heart beating at approximately 200 beats a minute; no cells, with the possible exception of the brain, are enclosed about by sheaths of connective tissue. Yet some cells show the dye far more quickly than others and in weak dilutions they get all of the dye.

If food matter may be assumed to follow the course of the stain, the situation is enlightening. Food is first digested extracellularly, that is, it is hydrolyzed sufficiently by enzymes poured into the digestive lumen to become soluble and permeable to the cells of the digestive tract, these passing the food through with no further change—at least, no further hydrolysis. It is then taken up by two systems of phagocytic cells, one of acid to neutral reaction being first to receive it, and then one of neutral to alkaline reaction. Or perhaps they take up two different types of food substance, just as the monocytes of mammalian blood digest with an acid reaction and the leucocytes with an alkaline reaction, each presumably selecting different types of foods, as they do different bacteria (Opie, '10).

Raw food materials are thus prepared for a second time in the phagocytic systems. Preparation undoubtedly means much more than simply the further hydrolysis that is so obvious in the large neutral-red bodies of these cells. Cladocera have no liver or gland cells (with the exception of the shell gland). The cellular secretions, hormones, so necessary as the regulatory stimulants and depressants of highly coordinated animals must emanate from these cells. It is this food prepared as a copious secretion which is taken up by the remaining body tissues—not the still comparatively raw foods passed from the digestive lumen into the body cavity. Such raw food material is available to all cells, but they do not receive it. Something akin to the opsonin idea may be indicated. (For discussion and bibliography, see Mann's chapter on the liver in Cowdry's "Special Cytology," 1928.)

Of prime importance, apparently, is the nutrition of the gonads, since these structures are first to receive the stain after the phagocytic systems. The excessive staining of the supporting elements of the gonad indicate without doubt their rôle as highly active digesting cells. Hydrolytic processes far exceed any synthetic processes, as often so much of the cell body is occupied by the hydrolytic apparatus that there is no room for synthetic and storage elements. The nature of these cells also suggests that they may give off prepared material into the body cavity as well as into the ovary.

In the 1:2,000,000 dilution it is approximately four hours before other tissues stain. Then the blood corpuscles, the cells of the digestive tract, the dermal cells of the respiratory appendages, and the shell gland are stained. All this happens long before the muscles show neutral-red bodies. Usually after the muscles the carapace cells and, lastly, the brain cells are stained. No single somatic cell in the body remains unstained. In the eye there is a brilliant neutral-red body in the unpigmented portion of each corneal cell, and the single ocellus has supporting tissue with definitely arranged neutral-red bodies.

Of what significance is the fact that the muscles, carapace, and brain cells are the last to stain? Continuing on the basis of the hypothesis suggested by the avidity of the phagocytic cells for the new food materials and the possibility of their preparing these made more valuable by secretions, an interpretation may be this. No cell of the body lives to itself; each gives off a secretion. The last cells to receive the stain are those that receive the most highly modified food substance. This seems appropriate enough for muscle and brain cells at least, that they receive the quintessence of prepared food. Their digestive powers are limited, as indicated by their minute neutral-red bodies, and their nutritive demands probably enormous. They therefore take in food so thoroughly prepared by the sum total of somatic and gonadal tissue digestion and synthesis as to be readily adapted, with but little further change, for the highest activity of the animal. Non-functioning or disfunctioning anywhere along the line will therefore be manifested in the altered efficiency of these two systems.

What of the germ cells, however? Is it not reasonable to suppose that they should receive the full complement of the secretions of the body? Perhaps they do. For the height of staining when every cell of the body is brilliant with its distinctive type of neutral-red bodies is of brief duration. The last cells to receive the stain lose it most quickly. Complete staining is retained for only about sixteen to eighteen hours. Within a few hours after this climax, the neutral-red bodies of the cells disappear in the reverse order in which they appeared, down to the cells of the gonads and the phagocytic systems. These persist. On the fourth day of staining the gonads are still brilliant. Usually by the end of the fifth and on the sixth day, only the neutral phagocytic cells show neutral-red bodies and these gradually disappear, the last to lose the stain being those around the posterior part of the digestive tract. The phagocytic systems and the gonads are not only the first to receive food materials; they also receive the ultimate secretions of all of the body cells.

That neutral red should thus follow the course of nutriment and secretions revealing, as it were, the physiological pattern of the animal, starting at the digestive tract, is amazing. Yet it is the only theory that will adequately fit the facts of this superb example of elective staining. One cannot say that it is simply in the order of greatest activity that the cells stain, in the face of the extreme activity of the heart and body muscles in Daphnia. It cannot be that neutral red is picked up as a waste material, for that would involve the young oocytes as scavengers. That the developing eggs store neutral red with food and that they remain unharmed thereby, developing and hatching as normal individuals, also capable of reproducing, has been ascertained beyond any doubt by keeping cultures continuously stained for many generations.

Evidence showing that neutral red truly accompanies substances entering cells and also leaves cells with their secretions is offered by various workers (Bensley, '12), the most recent being Winkelstein and Marcus ('29) on the excretion of neutral red into the stomach as a test for achylia gastrica. Neutral red is injected intramuscularly, 40 mg. in distilled water, into human patients. Within fifteen minutes, in normal persons the stomach contents show the presence of neutral red. In persons with hyperacidity neutral red appears in from five to eight minutes; in subacidity, in from twenty to sixty minutes, while in achylia gastrica in which no acid is secreted no neutral red appears in the stomach. Other digestive substances, the ferments, are almost always lacking when neutral red is not secreted—out of sixty such cases ferments were found in only thirteen. The authors conclude that neutral red is helpful in the study of normal and pathological gastric secretion: when stomach cells can secrete free acid and enzymes, they also secrete neutral red; if they cannot secrete free acid, they cannot secrete neutral red.

On the basis of this test, the very normal condition of carefully stained Daphnia is shown in the last stages of staining when the cells of the digestive tract—and it is particularly clearly brought out by the cells of the posterior region—

secrete neutral red into the lumen. These posterior cells in dilute solutions of the dye do not secrete the enzymes which combine with neutral red in the initial staining of the lumen. But their natural secretions, whatever may be their nature, are revealed when, receiving neutral red from the elaborated body fluid, they secrete it into the posterior intestinal lumen. This is final proof, not only that cells are not injured by neutral red, but that their secretory processes may be definitely revealed by the stain. Since neutral red produces no irritation nor unpleasant effects even in human subjects, the possibilities presented for its general use in physiological and cytological problems of nutrition, elaboration, and secretion are unlimited.

That the use of neutral red, however, may not be unattended by results of great significance must be predicted as the result of phenomena noted throughout this study. Neutral red seems to act as a stimulant of enzyme production, first, in hydrolytic processes and, secondly, in synthetic activities. (See parts on hydrolytic and synthetic systems.) The effects of such stimulation on an organism notably susceptible to metabolic regulators, the amphibian, have been noted.

In spite of difficulties in rearing both control and experimental animals, a preliminary report on the feeding of well-stained Daphnia to axolotl larvae deserves citation. The number of animals used and surviving was not large, but the results amply indicate what the neutral-red reaction may ultimately reveal.

Those animals (newly hatched) fed with stained Daphnia developed exactly as the controls fed unstained Daphnia, in size. After the first two weeks, however, they were noticeably darker, due to more pigment granules in the melanophores. A darkening of the usually transparent skull bones was especially noticeable. Approximately three months after hatching (cold-delayed development), these animals had developed well-formed hind legs and toes, whereas the controls showed no sign of posterior limb buds.

Pigment control in amphibians is attributed to pituitary function (Hogben, '24) and more generally to mitochondrial action (Rényi, '24). The development of limbs is well known to be a thyroid effect. (For literature and review of the subject, see Hogben's "Physiology of the endocrine system," 1927.) That a substance such as neutral red should occasion both of these changes points to some more fundamental metabolic factor than individual glandular control. A great part of the secret may lie in the interreactions between cellular hydrolysis and synthesis.

Résumé

The fluids of the digestive cavity of an animal, when they are of enzyme content, stain with neutral red. The fluids of the coelomic cavity never stain. It is known that proteolytic enzymes do not and cannot exist in coelomic fluid which is highly inactivating or 'anti-enzyme' in content. That neutral red is passed about freely in the coelomic body fluids is determined by the fact that cells bathed only in this fluid take neutral red into their vacuoles. The mere presence of neutral red in a body fluid or passing through a cell, as it does through the digestive cells into the body cavity, is altogether a different condition from the 'neutral-red reaction' where the stain is made visible by concentration upon enzyme surfaces.

The mechanism for the transfer of food material plus dye through digestive cells into the body fluid is unknown, but it is certainly not by way of the hydrolytic system of the cells. The digestive cells in Daphnia stain, as do other body cells in the higher animals, by way of the body fluid, from which they receive their sole food supply (Bensley, '29). The mechanism for the transfer of quantities of neutral red around in the body fluid in invisible form is an absolute mystery. Even when Daphnia are stained with concentrations of the dye which so load the cells with neutral red as to prove toxic to the organism, the coelomic fluid remains colorless.

That neutral red is passed about from one tissue system to another with the respective secretions of these tissues is indicated by the order of staining, but that such secretions are invisible unless they are poured into a lumen containing no 'anti-enzyme' is a fact.

Consideration of perfect neutral-red staining in a metazoan form brings out impressively the fundamental conception that each tissue cell is a unit unto itself. Each vital process is carried out independently, but the result produces the infinite coordination of an organism.

Notes

1. The description of staining in Daphnia applies primarily to the adult with ripening eggs in the ovary and usually with one set developing as embryos in the brood chamber. Variations, especially as to the intensity of staining, have been noted in young and senile individuals. These must be discussed in a separate paper.

2. Culex larvae have been studied in the same manner as Daphnia. They are not such favorable material, for all phases of the life history may not be followed. Also, in all the larvae so far examined the digestive tract has always been so full of dark material that it has been impossible to determine whether the reaction of the lumen is acid or alkaline. But the rôle of the phagocytic, or pericardial cells, is demonstrated. These cells, in long connected strands on either side of the heart with a terminal ring at the anterior end, are the first cells in the body to stain and they remain stained longer than any other cells.

Culex larvae are distinguished by great bands of 'fat-cells.' The reaction of these to neutral red is characteristic of embryonic staining where there are large masses of unformed material. Albuminoid or other protein materials seem to be more abundant than actual fat inclusions, and these bodies of various shapes and sizes assume staining of a brilliant acid color. A vacuole structure is not visible; it is presumed, however, that the cells have engulfed these embryonic materials and that vacuoles therefore exist. These stained inclusion masses are very close to the cell membranes, and beautifully stained blood elements line up along them and remain for indefinite periods. Very few blood corpuscles are noted wandering about in the body cavity and none are ever seen in the heart tube.

In Daphnia the carapace staining is delicate, two or three small neutral-red bodies to each cell. In Culex stained dermal elements are numerous and brilliant; the neutral-red bodies are small and tightly packed, especially in cells at the base of the bristles.

The cells of the digestive tract stain but very slightly, except in the region of the hepatic caeca, where numerous small neutral-red bodies crowd the cells. The developing muscle cells in the head of Culex stain beautifully and offer valuable material for the study of muscle histogenesis. The author has never yet seen the brain stained.

3. First-instar dragon-fly nymphs, Sympetrum vicinum, have also been studied. Immersion alone in a stained medium will not suffice for staining—they do not

take materials indiscriminately into the digestive tract as do Daphnia and Culex. They eat Protozoa one by one, evidently without swallowing much water. Consequently, Paramecia stained in a 1: 2,000,000 solution were added frequently to the water. The resulting dilution in the dragon-fly culture is so weak as to scarcely stain Protozoa in the culture. Therefore it is perfectly clear that neutral red is obtained solely with the food material, although there is really no question about this matter in any of the animals mentioned above. For instance, gills in the Daphnia, the tracheal appendage in the mosquito, and the rectal gills in the dragon fly never stain before the phagocytic systems.

There is a great deal of 'fat-body' about the stomach in the dragon fly, obscuring all details. The lumen, however, seems to become brilliantly red, a much deeper color than is ever noted in the Daphnia lumen. The intestine and the rectum never show any color. The pericardials are quite similar to those in Culex. They are arranged in pairs on either side of the heart, one group between each set of valves, and they end abruptly at the head end of the heart. The neutral-red bodies are large, comparatively few, and of a deep, rich neutral color.

Next in order of staining are the oenocytes. These lie in broad sheets in the ventral part of the abdomen. Their neutral-red bodies are extremely minute so as to be visible only under high powers of the microscope. They are numerous and scattered fairly evenly about the cytoplasm. They seem to vary in color in the same cell, but most of them are decidedly in the alkaline range.

It is difficult to determine when the fat-bodies in the thorax and anterior abdomen become stained, because of the dense color of the stomach. It is impossible to make out anything of the details of this dense, bright red staining. There is considerable dermal staining as in Culex, especially about the hairs and bristles.

4. Various oligochaetes, including Chaetogaster langi and Chaetogaster limniae, are successfully stained except for the muscles, nervous system, and nephridia. An undescribed element, a rather large type of cell scattered in the coelomic cavity, seems in every case to act as the phagocytic cell, being the first cell in the body cavity to stain. Later, the blood corpuscles, the epidermis, and the cells of the pharynx, crop, and intestine stain. (Staining of the gizzard cells has been described in the part on intra- and extracellular digestion.) Cells of the dorsal blood vessel usually stain with most minute neutral-red bodies.

5. Newly hatched Physa snails stain well. At first, there is considerable yolk material in the apex of the shell, which becomes a brilliant acid color. After a few days, this changes to the more neutral color, presumably as the liver cells are completely elaborated. It is impossible to distinguish the exact nature of the digestive lumen, which is for the most part hidden in the liver. Those portions visible show neither extra- nor intracellular digestion. The small cells of the foot, head, and tentacles have small neutral to alkaline bodies. The heart can be seen plainly and the heart-muscle cells have not been noted to be stained.

6. In some of the higher forms noted here, the insects, annelids, and mollusks, it seems impossible to achieve complete staining in any physiological dilutions. An explanation of this may be, not that insufficient stain passes into the coelomic

fluid, but that certain cells in the course of their handling of the stain keep it back or transform it so that it does not get into the secretions they give off. The ability of such cells to remain impermeable, through their vacuolar systems, to a foreign material such as neutral red may imply a higher degree of efficiency than those cells which are not only permeable but allow the stain to become involved, perhaps as some loosely held compound, with their secretions. The nervous system particularly seems to be protected. In higher forms this is undoubtedly the function of the reticulo-endothelial system, corresponding in function to the phagocytic and pericardial cells of lower forms.

7. These experiments show the ease with which neutral red combines and is released from combination with animal fluids. This probably accounts in great measure for its non-toxicity. Bechold ('19) says, "Such foreign bodies as become chemically fixed permanently injure the affected cell." Most cells seem to mobilize neutral red without difficulty.

8. Both protozoan and metazoan organisms dying during vital staining in the dilutions used in these experiments as a result of injury during handling lose all trace of color quickly. The spread of neutral red to the cytoplasm and the nucleus of the cell so often described in the literature of vital staining is never observed in weak dilutions. The exact significance of this, in contrast to behavior in toxic dye solutions, is of course not known. But it seems that autocytolysis after death is slow after dilute staining; very rapid after heavy staining, which is a powerful stimulant. When Daphnia are crushed, slightly broken, or injured by a chemical, the intestine lumen first loses color and then more or less rapidly the neutral-red bodies of the tissue cells, so that the organism becomes quite without trace of any staining. Death under these conditions may inactivate the enzymes along with other cellular processes. In the case of toxic staining, however, when the enzymes are so stimulated that they kill the cell, the situation may be such a one as Palladin ('26) describes: "When plants are killed without the destruction of their enzymes the physiological system of the cells appears to become completely disarranged with the destruction of the interrelations that obtain between the different constituents of the living cell."

It seems almost needless to repeat here the well-known fact that dead tissue, whether fixed or not, cannot be stained in very weak solutions of azine dyes. The methods of preserving neutral red in preserved vitally stained material are few. Rapid drying is one (Covell and Scott, '27). Formol and corrosive-sublimate fixation is another (Gardner, '27, and Adams and Shevket, '29).

THE DEVELOPMENT OF THE EGG AND THE PROBLEM OF DIFFERENTIATION

Among many species of rotifers, Brachionus urceolaris is a particularly favorable form, because it is rather broad, enabling one to see all of the organs, particularly the ovary; because the developing embryo remains attached to the parent body, and because the animal frequently attaches itself to

Daphnia, so that it may be observed for relatively long periods at a time.

In a 1: 1,000,000 solution of neutral red (see part on elective staining) the ovary is stained after two hours, provided the eggs are young. An egg with any considerable amount of grayish yolk never shows the slightest tinge of neutral red. Young eggs, however, are brilliantly stained. In the smallest and youngest the neutral-red bodies are comparatively few in number and exist in clumps. When the egg is larger, the neutral-red bodies are somewhat smaller and much more numerous, being scattered more and more evenly throughout the cytoplasm as development proceeds.

The pictures presented are altogether similar to those found by Parat ('26), Hibbard ('28), Nath and Hassain ('29), and other investigators of developing animal eggs. The universal occurrence of small vacuole-like bodies, increasing in number and decreasing in size as the egg develops, is recognized for practically all eggs described in the literature, however differently these bodies may be interpreted according to whether vital material has been investigated and according to the histological technique.

It is also recognized by Parat and by Hibbard that in the early stages these bodies are comparatively easy to stain supravitally, but that they become progressively more difficult to stain as yolk formation increases. It is not that the yolk globules mask the stainable bodies, but, as Hibbard explains, "During the building up of the yolk the aqueous droplets become more and more dehydrated and lose their capacity for being vitally stained." Thus the vacuoles disappear altogether in the ripe egg.

When it is possible to stain eggs by the natural process of feeding neutral red to the parent and watching the development of the egg in what is apparently a perfectly normal, if slightly stimulated, animal, the vitally stainable qualities of the egg acquire greater significance.

One mature egg at a time is produced by Brachionus, the fate of the two or three other eggs noted at equal stages of development in the young ovary being to contribute, in the rôle of nurse cells, to the single developing egg. Young ovaries are brilliantly stained on the first day; the next day, the grayish, yolk-laden egg appears with no sign of staining; by the third day, this egg is usually laid and is attached to the body. It shows no neutral red.

As long as the egg has not cleaved and at least until the fourth or fifth cleavage, the egg being holoblastic, there is no sign of stain. But in the morula stage staining becomes apparent, each cell with a circumnuclear ring of delicate and numerous neutral-red bodies. This stage is very transient, lasting perhaps but two divisions (this has not been definitely determined), and there is no further sign of neutral red until the young individual emerges and begins to feed.

It must be emphasized that no eggs with any considerable amount of yolk within the ovary nor eggs that have been laid at the time the parents were stained ever show any staining until they become feeding individuals. Such eggs are not permeable to the stain, at least to the dilutions in which the organisms live and thrive. This means that the reappearance of stain in the embryo is a true manifestation of the initiation of a definite functional activity in development.

The above facts resolve themselves as follows: Young eggs in the ovary are absorbing food material from the medium of the body cavity and no doubt some from the few supporting cells of the ovary in this form. This they hydrolyze in an extensive system of vacuoles by means of enzymes with a decidedly acid reaction. As digestion and storage progress the vacuoles become more numerous so that every part of the cytoplasm contains them, but they also become smaller, as absorption is becoming slower. Metabolism must slow down, with the accumulation of vast stores of food materials, food taking thereby becoming laborious, hydrolyzing enzymes being sparsely contributed when the synthesizing processes of the mitochondria are utilizing more and more of the available enzyme supply; until finally the egg becomes an inert mass, anabolic processes having stopped. As such a mass, the egg is expelled from the organism.

In these ordinarily parthenogenetic eggs the stimuli of the new medium, lessened osmotic pressure, more oxygen, etc., seem sufficient to start development. The first few divisions of the egg occur without any sign of the stored stain appearing; then neutral-red bodies appear. The author interprets this as the initiation of differentiation. Yolk material in this egg is of uniform consistency as far as is visible. The first few divisions therefore produce cells identical as to substance. Cellular differentiation involves primarily chemical differentiation. A uniform substance must be hydrolyzed and resynthesized before differentiation is possible.

Cells varyingly exposed to water taken into the egg, to the oxygen of the exterior or interior layers of the egg, to CO_2 distribution, to slight alkalinity or acidity effects, to various permeable salts from the medium will hydrolyze with corresponding variation, as hydrolytic processes are particularly sensitive to such environmental factors. The degree to which otherwise identical cells are subjected to these factors, depending upon their position within the egg, will determine the degree and perhaps the nature of their hydrolysis.

Only a small degree of hydrolysis is necessary at this early stage to affect great changes, and the neutral-red reaction seems to be of brief duration in this case. When the young individual emerges and feeding is begun, staining is a repetition of the process described in the parent.

Daphnia is likewise an extremely favorable organism in which to observe vital staining in the developing eggs and embryos, as the ovarian string presents a series of ripening eggs, and the eggs are laid into the brood chamber in which they remain until the young become fully formed. Ovarian strings, described by Claus ('76), have young eggs and nurse cells, indistinguishable from them in the early stages, at one end of the organ and large, enormous vacuolated cells, 'Eierbehälters,' toward the ripening end. Each maturing egg absorbs into itself the total substance of three adjacent cells, and from this substance and from contributions from the Eierbehälters yolk is accumulated.

(For details as to time of staining, etc., in relation to the whole organism, see chapter on elective staining.) The young cells, egg and nurse, stain alike with clusters of minute neutral-red bodies. Somewhat later, the egg cell is distinguished from those that are to lose their identity by staining with a uniform distribution of small neutral-red bodies, while the nurse cells no longer show any. They have ceased functioning as far as food taking and hydrolyzing are concerned. During the absorption of these cells, the egg neutral-red bodies increase in number and decrease in size. Yolk begins to make its appearance as small green spherules (in animals well fed with lettuce). The egg lies among the large 'Eierbehälters.'

These cells with their enormous vacuoles produce great blobs of color out of proportion to all other neutral-red bodies in the entire animal. They are extremely brilliant and acid in reaction. No nuclei nor other cellular constituents are visible at this stage. Such a degree of hydrolysis undoubtedly softens the cell membranes to such an extent that the cellular contents, or rather the vacuole contents, escape, forming a medium for the ripening egg.

Coincident with yolk formation, the neutral-red bodies of the egg decrease rapidly in size until they are no longer visible. Thereafter, the egg shows no sign of staining as long as it remains in the ovary. Then the mature egg is squeezed through the short oviduct into the brood chamber, where it rapidly assumes an oval shape. In one variety of Daphnia pulex the eggs are laid singly at three-hour intervals by means of an oviduct in the anterior of the ovary, the older end, and so pass into the brood chamber at a point just behind the heart. In another variety of this species, otherwise distinguished by minor internal differences only, the eggs remain in the ovary until all (usually about six) are mature, at which time they pass through oviducts situated one on either side of the intestine at the posterior of the ovary, which is just at the bend of the intestine.

In the first variety it is noted that after each egg has remained for three hours, approximately, in the brood chamber, a superficial cellular layer at least one cell thick has cleaved. At this period neutral-red staining begins, appearing as a red film upon each yolk globule, so that by the time a second blue-green egg is laid into the chamber, the first is in great contrast with the neutral red superimposed upon the blue-green yolk globules of the center.

Again it must be emphasized, as for the rotifer egg, that no egg in the ovary with any visible amount of yolk will stain unless it has been stained in earlier stages; that maturing eggs present at the time the organisms are subjected to the stain will not stain nor will eggs in the brood pouch ever take any dye from the medium. Only young absorbing eggs hydrolyze to any great extent, and therefore these only show neutral-red bodies. Eggs which are undoubtedly taking in quantities of secretion from the Eierbehälters do not hydrolyze this substance to any visible extent—it is perhaps a highly specific substance supplied practically ready for synthesis in the egg in much the same manner as the grasshopper apical cell supplies secretions requiring little hydrolysis to the spermatogonia. (See part on the development of the sperm.)

It is clear, then, that reappearance of stain in an egg within the brood chamber can have but one meaning—the reappearance of hydrolytic activities. Early cleavages have occasioned no hydrolytic functions and the cells produced are presumably all alike chemically as they are visibly. But somewhat later, yolk material ceases to be utilized in such monotonous fashion. In some way water is made available to initiate hydrolytic processes on the surfaces of the yolk globules and the cells now formed from this transformed material will be different chemically as well as visibly from those primarily divided off from undifferentiated stuff.

No cells in the developing Daphnia embryo show any neutral-red bodies, and many superficial cells are distinctly visible throughout development, so that this does not seem to be a mistake in observation. Staining is confined to the surfaces of the original nutritive material. It is uncertain as to whether fat-globules are affected. Fat is certainly not all utilized in embryonic development, for the phagocytic cells in the late embryo and the young individual each have one or more large globules of fat which do not stain with neutral red.

There is no sudden disappearance of the neutral-red reaction in Daphnia embryos as in the rotifer. Perhaps the reaction continues to the end of yolk utilization; the original red color over the blue-green globules changes and the yolk becomes more and more purple. (These stained embryos must always be compared with the unstained controls, for color variations in Daphnia eggs are numerous. It is best not to feed much lettuce and to rely upon sheep manure as the main food supply. Such eggs will be dull-colored and are much more favorable for showing up the neutral-red reaction.)

The embryo of Daphnia then presents the phenomena of differentiation in the egg of the superficial cleavage type. The cells, not having any yolk material, have nothing to break down and therefore show no hydrolytic processes. But at the yolk surfaces hydrolysis is constantly taking place; it is possible that the products thus formed are continually different, as the internal egg environment changes with every division. The possibility of infinite differentiation down to the last molecules of yolk may thus be indicated under the microscope.

Conklin ('24) states:

> It is important to realize that the ultimate causes of differentiation must be found in chemical and physical phenomena, but it is equally important to remember that the last step in such an investigation cannot be taken before the first and intermediate steps, and the first step is the study of morphological and physiological differentiation under normal and experimental conditions.

The neutral-red reaction promises considerable aid in the study of normal differentiation whenever it is possible to stain ovaries in situ in such manner as to injure in no way the organism, so that the developing eggs and embryos may be traced with certainty as to their normal development with at least one process clearly manifested by a color reaction of known significance.

Parat ('27) describes almost these same phenomena in the eggs of some medusae and in nudibranch molluscs with supravital staining. The young eggs stain; ripe eggs do not stain, and there is no staining until the blastula in Chrysaora and the gastrula in the nudibranchs. His interpretation is: "Il représente pour nous la naissance de novo du vacuome par suite de l'hydrolyse des plaquettes vitellines formées dans l'oeuf par un processus exactement inverse."

It is thus probable that such phenomena are of universal occurrence and that they may be demonstrated correctly when the neutral-red stain is carefully used. There are numerous examples in the literature of the staining of ripe, unfertilized and fertilized eggs where the dilutions of the dye are either not mentioned or are revealed as extremely toxic concentrations. Until it is positive that stained eggs, either vitally or supravitally treated, will develop normally in comparison with controls, the reactions may be discounted. But when developing eggs are stained during their normal absorptive period in dilutions which do not harm the adults, the ensuing manifestations offer a valuable clue to the problem of differentiation and a tool for the experimental embryologist.

Note

Cyclops eggs are holoblastic. They stain as such—each blastomere showing neutral-red bodies. Staining persists through many cleavages (exact number not yet determined).

THE DEVELOPMENT OF THE SPERM AND THE PROBLEM OF FERTILIZATION

When male Daphnia are stained in vivo, none of the germ cells stain. The testis is a slender sac immediately ventral to the digestive tract (Weissman, '74). The supporting cells are small; they stain brilliantly with tightly packed neutral-red bodies. Neither in the fully matured spermatozoa, free in the lumen, nor in those attached to the walls adjacent to the supporting cells has any sign of stained bodies appeared.

In the grasshopper the blood elements stain in vitro in a 1:1,000,000 solution within at least ten minutes, so that it is presumed that this dilution is a fairly harmless solution. When grasshopper testes, Chortophaga v. and Melanoplus d., are treated with such a dilution in Ringer-Locke, blood cells adherent to the follicles become brilliant. Just as quickly and even more brilliantly, the apical cell of each follicle develops color in all of its numerous branches and its dense cell body. Also all of the supporting elements of the follicles show neutral-red bodies. But further than this there is no staining in the preparation as long as it survives—no germ cells stain.

The degree of difference between the somatic cells and the germ cells is brought out by increasing the concentration of the dye; the blood corpuscles and the apical cell can be manifestly injured long before the germ cells show any sign of neutral red. At a concentration of 1:50,000, or better, 1:40,000, staining in various stages of the germ cells occurs. The neutral-red complex so revealed is quite in accord with the findings of Lewis and Robertson ('16) for Chorthippus, and need not be reviewed here.

These staining phenomena may be interpreted in the light of the neutral-red reaction as follows: The picture presented is one of intensively secreting cells in contrast to non-secreting cells. Secreting cells are rich in neutral-red bodies, for they take in quantities of material from the plasma, hydrolyzing more than would be sufficient for the needs of the individual cells. The synthesizing apparatus, the mitochondria, may be almost obscured by the packed red granules and storage granules are very inconsiderable. Secretions must therefore be rapidly released into the follicular medium. It is clear that the somatic cells—blood corpuscles, supporting cells and, preeminently, the apical cell—are 'nurse cells' for the testicular follicles.

The number of these somatic nurse elements is small, considering the number of germ cells in each follicle; but on the other hand, the food requirements of the spermatogonia are not heavy—they are not storing cells nor secreting cells. It may be, even, that the single, large, branching extremely rich apical cell may be the immediate source of a specific secretion which must supply each cell in the follicle, the supporting cells supplying a more general secretion.

At any rate, it is in the region of the apical cell that the germ cells take in nutriment, for it is in this region that the spermatogonia show the usual cytoplasmic relationships. Here the neutral-red bodies, although few, are somewhat scattered and the mitochondria are free and active. To some extent in the first spermatocyte and quite markedly in the second, the mitochondria show the Nebenkern-formation process and anabolic functions must have ceased. The secretion from the apical cell is probably so perfected as to require little hydrolysis.

Important, however, as the spermatocyte vacuoles must be in receiving the small amounts of food necessary for the maintenance of the cells during division, they seem to acquire far greater significance in the process of sperm formation. It is the vacuole apparatus which goes to form the acrosome which composes usually the tip of the spermatozoan head (Monné, '27; Poisson, '27; Voïnov, '28). Lewis and Robertson do not find an acrosome in Chorthippus, but they find two neutral-red bodies on either side of the sharply pointed nucleus at the tip of the head. The French cytologists, Monné in the molluscs and Poisson and Voïnov in Hemiptera, show the identity both in vital and in fixed preparations between the modified neutral-red bodies and the acrosome. The acrosome is primarily the fusion of the neutral-red bodies, dehydrated and concentrated into a dense structure such as is found in no other tissue cell.

If the acrosome, then, is directly derived from the neutral-red bodies and if the theories developed in this study concerning the nature of the neutral-red reaction have any universal significance, they should, to be consistent, explain the nature of the acrosome as a modified digestive organ. This would involve a theory of fertilization based upon suggestions offered by the neutral-red phenomena. As a matter for speculation, this theory is presented.

Now it has been found by Bodine ('29, in press) that the membrane (chorion) of Fundulus eggs stains vividly with neutral red both before and after fertilization. Other marine eggs also adsorb neutral red (Clark, '17; Svetlon, '29). The author has found axolotl eggs adsorbing enough neutral red from a 1:5,000,000 solution for the outermost jelly film to become decidedly pink. Hermaphroditic snail eggs, L. palustrina, do not stain. Eggs released from the proglottid of the dog tapeworm into Ringer-Locke stain brightly. Parthenogenetic eggs show no membrane staining in Daphnia and rotifer eggs. Stripped Rana eggs precipitate neutral red immediately as sharp orange crystals adherent to the membrane, giving the eggs a decidedly fuzzy appearance in dilutions of from 1:20,000 to 1:40,000. Such frog eggs are easily fertilized and the first two cleavages appear within four hours (temperature about 19°C.). Development to the tadpole stage is normal.

Then the egg membrane is also a digestive organ? It is probable that the mature, tightly packed egg dehydrates itself by exuding from its surface the excess of the rich synthetic enzymes so abundant during yolk formation. Mitochondria and pigment granules often gather at the surface of eggs.

If pH, tonicity, temperature, etc., are optimum, the enzyme, in the oviduct in some cases, in the external medium in others, becomes hydrolytically active just as it would in watery cell vacuoles. Such a substance would be delicately specific for cells of the same species coming into range of it and could therefore exert upon the membranous covering of the sperm head a weakly cytolytic effect. Under natural conditions this is probably no more of an effect than a slightly stimulated enzyme production would have upon its own vacuole walls—it renders them more permeable. (See part on cellular hydrolytic system.) Thus, with the sperm membrane, or micropyle, softened and made permeable to water, the dehydrated acrosome becomes hydrated. Activation of such a concentrated substance is probably sufficient to destroy the whole sperm-head membrane, or the acrosome substance is released in one spot. Popa ('27), without apparently being aware of the nature of the acrosome, demonstrates this reaction by using strong solutions of Janus green and neutral red upon Arbacia sperm. In this case even the Nebenkern is involved and seems to become hydrolyzed if the reaction is long continued.

Such a reaction on the part of the sperm may simply bury it in the egg membrane, where it slowly digests itself, but if it touches a spot on the egg which is particularly vulnerable, such as the old food-vacuole canal or the point of attachment in the ovary or at the nurse cell, the sperm enters, widening the way with the digestive effect of its flowing enzyme. In some eggs, however, it may enter at any point and so digest its way into the cytoplasm. One should be able to predict whether an egg is normally monospermic or not, from its ovarian nutritive connections. In this way polyspermy may result unless the egg, at the moment of injury of the membrane by the first penetrating sperm, reacts with the lifting of the fertilization membrane, which must have 'anti-enzyme' properties.

The idea of the digestive properties of the sperm is not new; Gies, in 1901, considered the problem; Champy, in 1923, suggested it. Robertson, in 1912, extracted a substance from the sperm and noted its agglutinating and cytolyzing effect upon the egg; Hibbard ('28) asks: ". . . . l'acrosome est-il doué d'action enzymatique?" and after extracting such enzyme from the sperm by ether, concludes, "Une action digestive du spermatozoïde sur les membranes de l'oeuf a été mise en évidence; il est probable que le ferment est contenu dans l'acrosome."

Glaser is responsible for most of the ideas of the enzymatic nature of egg secretion. In 1913, he notes a synthesis of an ester accomplished by egg secretion; in 1922, the hydrolysis of higher fats; and in 1923, the striking relation of copper in proteolytic enzymes to copper in egg secretion.

But the idea of the egg cortex and the sperm acrosome bearing fundamentally identical substances is suggested only by the neutral-red reactions of these parts. It appears quite consistent with all of the known facts of fertilization. The egg slowly but continuously (three days in Arbacia; Lillie, '14) secretes a weak but active substance dissipated in the medium. This substance is sufficient to soften the sperm membrane, or more probably to open a micropyle (Popa, '27), thus releasing a powerful hydrolytic agent. If the impetus of this release can be expended upon the egg cytoplasm, the sperm nucleus is saved within the egg. Failure means autodigestion within the egg jelly or agglutination.

Lillie and Just ('24) state: ". . . . it is necessary to postulate both a special substance borne by the spermatozoan and also one borne by the egg which interact in fertilization." The neutral-red reaction may indicate that there is but one substance, a proteolytic enzyme common to both cells, excreted in the dehydration of the egg as a superfluous substance, and released from an inactive state in the sperm.

Those experimental processes prohibiting fertilization are facts which might be explained on this basis. Repeated washing of the unfertilized egg (Lillie, '14) only prevents the accumulation of the secretion at the surface, where it is usually held by the egg jelly and sometimes stabilized by a pigment, as in Arbacia (Glaser, '21). Washing plus the use of charcoal, which adsorbs the secretion, hastens the entire utilization of the secretion (Glaser, '21). Any known proteolytic enzyme would behave similarly.

Richards and Woodward ('15) state definitely that the effect of x-radiation upon egg secretion is of the same quality and degree as the effect of radiation upon ferments. They state also that agglutinating efficiency, like pepsin, varies with the square root of the concentration.

Blood and coelomic fluid inhibit fertilization. This is undoubtedly why Hibbard ('28) did not note staining of the membranes in the dissected-out Discoglossus egg and why exceedingly heavy insemination was necessary to effect fertilization. But "both eggs and sperm exposed to coelomic plasma and washed free of it again are fertilizable" (Lillie, '14). It is well recognized from the work of Metschnikoff ('05) and Opie ('10) that these fluids contain an inhibiting substance which inactivates cellular enzymes when they are oversecreted or liberated by cell destruction in inflammation. Such an inactivating effect upon both the egg and sperm cellular enzymes might indicate the common nature of their secretions. Lilie also shows that egg secretion acts in exactly the same manner as leucocytic secretion by the following experiment: "If a sample of plasma rich in inhibitor be divided into two parts and one part be saturated with egg secretion it is found that this portion has entirely lost its inhibiting properties." Ordinary somatic-cell vacuolar contents and egg secretion may be surprisingly alike. To be sure, 'eggprotease' and 'spermprotease' may differ from each other as much as 'leucoprotease' and 'lymphoprotease' do.

Concentrated egg water in a hypertonic solution fertilizes the egg (for the latest reference, however, to this subject, see Just, '29) and an extract of sperm likewise fertilizes the egg (Robertson, '12; Sampson, '26). Is not a common substance indicated? Likewise, egg extracts cause a strong swelling of the head of the sperm and ultimate cytolysis (DeMeyer, '11), and vital dyes will stimulate the sperm heads into autodigestion (Popa, '27). Both egg and sperm secretions may therefore exert digestive influences, the one upon the other, while each may exert the same effect upon itself.

CO_2 suppression (Cohn, '17), KCN paralyzation (Loeb, '14), acid inhibition (Smith and Clowes, '24), and the alkaline favoring effect (Hörstadium, '23) may all be interpreted as effects upon enzyme action (Bayliss, '19), and particularly cellular enzymes (Opie, '10), as well as in connection with the facts of cell-vacuole physiology as demonstrated by Lewis and Lewis ('24) and by Chambers ('28).

With enzyme activity so strongly suggested as the keynote to fertilization phenomena, it may be well to review the general physical and chemical properties of egg secretion with the corresponding properties of enzymes. For the most part the data on egg secretions are from Lillie and Just ('24) and the data on enzymes from Bayliss ('23).

Egg secretions	*Enzymes*
Colorless (often protecting pigment)	Colorless
Do not pass Berkefeld filter	Do not pass Berkefeld filter
Pass hardened filter-paper	Pass some hard filter-paper
Non-dialyzable (see note 5)	Non-dialyzable (see note 5)
Optimum temperature	Optimum temperature
Heat resistance, no definite figures, thought to be near 100°C.	Heat resistance, varying figures: 70–75° (Bayliss) 60° (Bayliss) Over 80° (Bayliss) 100° (Palladin, '26) Boiling for two minutes—diastase Boiling (Bayliss, '14; Sundquist, '25)
Stable—disintegrates only after months in water	Stable
Colloidal	Colloidal
No clear response to protein tests	No clear response to protein tests
Contain C and N	Contain C and N
Contain Cu	Contain Cu
Precipitated by $(NH_4)_2SO_4$	Precipitated by $(NH_4)_2SO_4$
Precipitated by strong alcohol	Precipitated by strong alcohol
Optimum pH	Optimum pH
Specific for certain species	Specific for certain proteins

Glaser ('23) expresses somewhat the still current opinion regarding the problem of fertilization as a most complicated process; also stating: ". . . . and if one thing is more certain than another it is that the major classes of evidence have not yet been handed in." That the evidence may be in and that it may be immediately available for interpretation as fundamental enzyme phenomena is strongly suggested by the rationale of the neutral-red reaction as applied to the egg and sperm.

Notes

1. Stripped eggs will show the neutral-red reaction in contrast to eggs dissected out as echinoderm eggs commonly are. The reason is apparent as due to the inactivating effect of contact with coelomic fluid, which always necessitates the heavy insemination of such eggs. Eggs may stain long after fertilization. This is because the surrounding egg jelly is soaked with secretion, although no more is produced (Lillie, '14). In axolotl eggs staining of the membrane persists until the egg hatches and the empty pink membrane is left behind.

2. Eggs without extra membranes, jelly, or a protecting pigmented secretion will probably never show the neutral-red reaction any more than the emptying red-stained vacuoles of Protozoa show any sign of color instantly after passing the cytophyge.

3. It is not supposed that, in general, extracts from sperm or egg membranes will immediately digest egg white, casein, and such foreign proteins. No doubt, these extracts will prove as nicely specific as the delicately adjusted plant proteases which will act only upon the proteins of the same plants (Blagoveshchenski, '21). But with suitable adjustment it is predicted that such extracts will hydrolyze. Kiesel ('24) found that the bladder liquor of Utricularia vulgaris, which digests small insects, would not digest fibrin, egg white, nor gelatin. But Adowa ('24), working with the same plant, found that saline and HCl extracts digested these substances, and furthermore that HCl increases β-protease activity, while $CaCl_2$ in neutral solution increased α-protease activity. This situation is carefully worked out by Hugounenq and Loiseleur ('25), who experimentally alter the functional groupings of pepsin and trypsin so that one behaves like the other; and they also alter proteins so that, e.g., albumin which has been acetylated or treated with formol will be digested by trypsin. They conclude that the colloidal support of both trypsin and pepsin is similar and that it is the mineral element, or the functional groups, which differ. Neutral red may indicate that the colloidal support of all proteolytic enzymes is common.

4. Some sperm are evidently but partially dehydrated and so dissipate their secretions and lose fertilizing ability within a short time (Lillie, '15); others must be so thoroughly dehydrated as to be completely inactivated, prohibiting any possible secretions. In the amphibian, Eurycea b., ripe, motile spermatozoa are stored in the female spermatheca long before the eggs are laid (Koehring, '25).

5. There is some question as to the dialyzability of both egg secretions and enzymes.

6. The intimate connection in most cells (see chapter on the morphology of the neutral-red bodies), and especially in the egg and the sperm, of neutral-red staining with structures of high lipoidal content always suggests something more complicated than independent proteolytic activity. A very partial answer may lie in Marston's ('26) "On the acceleration of enzymatic synthesis of proteins by lipoidal emulsions" and Korsakova's ('10) "Ueber den Einfluss der Zelllipoide auf die Autolyse der Weizenkeine."

7. It is well known that frog eggs must be laid when ripe. If they become 'overripe,' they can no longer be fertilized when stripped. If they remain in the oviducts they are gradually resorbed into the organism, so that the oviducts

are cleared of eggs and the frog seems to suffer no ill effects. What process other than some type of hydrolysis can account for the disappearance of 2000 complicated products of synthesis? A mature egg seems to have gone just beyond the climax of synthesis in the direction of reversal of the process, with the cortex or some portion of the cortex as the site of the reaction. Such a process could proceed rapidly and unhindered in an oviduct containing no 'anti-enzyme.'

THE MORPHOLOGY OF THE NEUTRAL-RED BODIES AND THEIR RELATIONS TO THE GOLGI APPARATUS AND THE MITOCHONDRIA

In Amoeba and in Paramecium the membranes of the neutral-red bodies are delicate and not at all prominent; they are simple lines. When osmic-acid fumes are used on these forms (technique of Lewis and Lewis, '24, and Covell and Scott, '28), the food vacuoles become no more sharply outlined. Amoeba proteus especially are so perfectly preserved by osmic fumes with no color changes or blackening of any part that after days of osmic exposure they preserve the illusion of life. In Paramecium the food vacuoles are just as delicately preserved, although the parabasal bodies blacken considerably. There is no lipoidal substance connected with the vacuoles in these forms other than the constantly shifting mitochondria.

It is among small forms such as Amoeba guttula, Amoeba choas, Nuclearia simplex, Hyalosphenia, Chilomonas, etc., that the outlines of food vacuoles are characterized by actual sculpturing. Such variations in the thickness of the walls of the vacuoles are invisible without staining, and with staining they are accentuated by polychrome differences. Red and orange areas predominate on the inner surfaces; blue, purple, and black on the cytoplasmic side, and rarely there are the brown tints found by Sabin, Cunningham, and Doan ('25).

The phenomena were first noted in vitro, in this study, in grasshopper blood elements and were thought to be some effect of the medium—lack of oxygen or too heavy staining. But with Protozoa in a natural medium and stain in which the organisms survive indefinitely, vital polychrome staining of the vacuole walls is noted to be just as characteristic for some forms as the simple pale red membrane is for others.

When cells with vacuoles of this type are exposed to osmic fumes, the vacuole walls blacken immediately and remain blackened during further treatment. In such cells, therefore, the osmiophil or lipoidal substances are plainly visible, with staining, during life. In other cells with more or less dense, clear, and uniformly staining vacuoles there is no such structure manifest during life; nevertheless, osmic acid brings out the existence of this structure, usually as the very characteristic Golgi apparatus. Some insect male germ cells are of this type. Covell and Scott ('28) and Covell ('29) show that nerve cells and pancreas cells have clear, homogeneously staining vacuoles which take on a rim of black in osmic fumes, thus demonstrating intimately related lipoidal material.

Thus, while it is undoubtedly universal that all animal cells possess watery vacuoles with an affinity for neutral red and that these vacuoles are osmiophobic and chromophobic with histological reagents, the osmiophilic and chromophilic substances in the cytoplasm usually closely accompanying the vacuoles may be made visible during life, or may be brought out only upon the death of the cell, or may be altogether lacking in the vicinity of the vacuole.

Now in Amoeba proteus the mitochondria seem in no way to be intimately concerned with the food vacuoles. Amoeba verrucosa shows a cap of slowly moving mitochondria on one side of new vacuoles. (See Nath, '28, for mitochondrial cap on vacuole in spider oocyte.) In many ciliates the mitochondria appear rapidly at the vacuolar walls, bombarding the membrane and remaining with the vacuole a considerable period. (See part on cellular synthetic system.) Also Nath's ('28) young spider eggs show that relationship. In some hypotrichs, e.g., Histrio, mitochondria appear quite stationary upon the vacuole membrane as red-stained beads; in some small Amoebae, Hyalosphenia, Actinophrys, and Actinospherium, the mitochondria, whether they be few or many, become purple or blue during neutral-red staining and those near the food vacuoles approximate so closely to the purple or blue-black walls of the vacuoles that it is difficult to distinguish them. This is especially prominent in grasshopper blood cells, the chromophils (paper on grasshopper cells in preparation); the deep blue mitochondria slowly weave in and out among the varicolored vacuoles and when they become quiescent, adhering to the vacuoles, they may not be distinguished from the dark-stained vacuole wall.

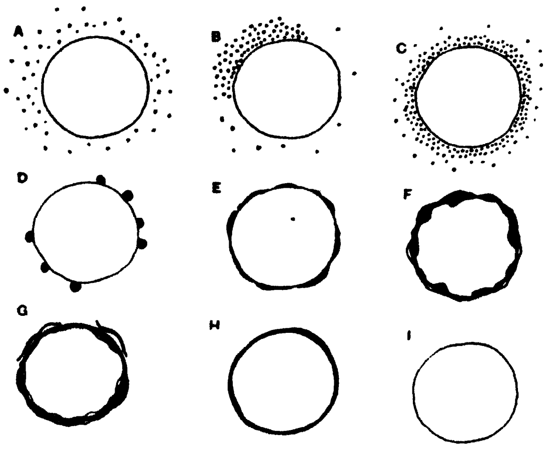


Fig. C The suggested evolution of the Golgi apparatus: a. Amoeba proteus. No lipoidal substance related to the vacuole; the mitochondria altogether independent of the vacuole. b. Amoeba verrucosa. A cap of mitochondria gathered about each new vacuole. c. Paramecium. Mitochondria clustered in great numbers about newly forming vacuoles and persisting during the greater part of the history of the vacuoles. Some may be adherent to the vacuole membrane. d. Histrio. Mitochondria in beads adherent to the walls of the food vacuole. Typical for many hypotrichs. e. Hyalosphenia. Mitochondria adhering closely to the vacuole membrane and imparting to this structure the mitochondrial reaction to neutral red—in this case, a blue purple. f. Chilomonas, Mastigamoeba, Amoeba guttula. Mitochondria in the cytoplasm not stained, although they are more easily visible during the neutral-red reaction. Vacuole walls very heavy and densely stained, the outer region blue, purple, and black. g. Grasshopper chromophil (developing blood cell). Vacuole walls heavily stained. Mitochondria appear dark. They wind closely about the vacuoles and soon (observed in vitro) adhere to the membrane, thereby becoming indistinguishable from the blue to black and purple wall. h. Human monocyte. After several hours of neutral-red staining, a black rim appears about the neutral-red body. i. Any non-amoeboid vertebrate cell. The vacuoles are homogeneously stained and the membrane is not differentiated. No lipoidal substance is demonstrated by neutral red; intimately related lipoidal substance is demonstrated by osmic acid and by all Golgi technique.

In other cells, for example human monocytes, there is no heavily stained vacuole wall visible during initial staining, but after some time, from twelve to twenty-four hours, a delicate dark ring, almost black, appears as, or connected with, the vacuole wall. Many of the Lewis and Lewis ('26) figures show these black rims. There seems to be no relation between these walls and any mitochondria in the cell. Lastly, in most fixed-tissue vertebrate cells, presumably the highest developed cells, there is not only no differential staining of the vacuole walls, but no connection at all between the vacuoles and the mitochondria; and, in fixed and stained preparations, the vacuole lipoids or Golgi stuff are quite different from the mitochondrial material (Covell, '28).

The facts are enough to suggest an evolutionary trend in the lipoids of cells and to advance the hypothesis: The Golgi substance is a derivative of the mitochondrial complex. The idea is not new; Parat ('26) suggests: ". . . . des phénomènes de tension superficielle se manifestant surtout par l'accolement souvent très intime du chondriome et du vacuome. Le chondriome peut être à la fois condensé, hypertrophié et accolé aux vacuoles."

That the enzymes borne by the mitochondria are essentially the same as those in the cell vacuoles is indicated wherever the vacuole walls are readily permeable to neutral red. It is but one further step to supose that the mitochondria are not only bearers, but the actual producers of the proteolytic enzymes. When mitochondria become concentrated upon the surface of the vacuole, they may secrete into the vacuole; scattered throughout the cytoplasm, they utilize materials for general cell use; concentrated at the secreting pole of the cell, they elaborate superfluous material into reserve substances such as the zymogen granules which are excreted from the cell upon opportunity; having completed intensive functioning, they may migrate to the surface, as in the highly nourished egg, and secrete a diffuse product; gradually atrophied, as in the malnourished spermatozoon, they round up into a Nebenkern which perhaps becomes altogether functionless;

the substance remaining with the acrosome retains the power of secreting a concentrated product into the hydrated vacuole.

Mitochondria change as to fixing and staining reactions in such modifications as the parabasal bodies (Causey, '26) and the Nebenkern (Bowen, '22). No more extensive changes would produce the Golgi substance (Gatenby, '19). There is a possibility even that there is no chemical change involved in any of these transformations, the differences being as Hugounenq ('27) suggests: "Substances may be chemically identical but have different colloidal structure. The entity, the colloidal particle positive ion may be regarded as the catalyst. The individual arrangement of molecules determines the properties of the diastase." Such rearrangement of molecules would be very likely to influence staining and fixing reactions and so account for the caprices of mitochondrial and Golgi technique. On the other hand, Fauré-Fremiet's ('10) suggestions may be more correct and the various reds, yellows, and deep blues of neutral red may be indicators of different types of lipoids.

Parat comes close to this view of the fundamental nature of the mitochondria in 1928:

> Et nous serions alors amène à envisager dans la cellule, deux categories de chondriome: le chondriome de la zone de Golgi ou chondriome actif, et le chondriome de cytoplasm, non couplé avec le vacuome, auquel on pourrait donner le nom de chondriome ordinaire.

Such views are in direct opposition to Duesberg ('26): "Ainsi, un premier point est acquis: l'appareil de Golgi est distinct des mitochondries," to Cowdry ('24), and to many others. However, with the thought of the enzymatic nature of the mitochondria and the undoubtedly somewhat similar nature of the Golgi material in mind, it seems decidedly worth while, even in the face of the comparatively small amount of evidence presented so far, to adopt, tentatively, a fresh point of view and to start anew on the analysis of the lipoids of the cell with their physiological manifestations in mind.

In Protozoa, although evidence suggests strongly the connected nature of the vacuoles in non-amoeboid cells, such connections are not actually visible. In the Daphnia neutral phagocytic cells, however, there are neutral-red bodies in more or less continuous chains. An entirely circumnuclear chain has never been noted—there are two, three, or four masses with the individual vacuolar constrictions barely visible (see fig. 74, however). The vacuoles are plainly confluent. What may be their relation to the outside or to the vacuoles of connected cells is not at all manifest.

The corresponding systems, the pericardial cells, in the mosquito and dragon fly studied in vivo reveal only spherical, apparently unconnected vacuoles. But in grasshopper pericardial cells, even under in-vitro conditions, connections between the vacuoles, or at least branches into the cytoplasm, are sometimes indicated. That is, the vacuoles are often not spherical, but taper finely at both ends.

No doubt, further study of all types of in-vivo material will reveal numerous examples of vacuoles so closely connected that the short, wide connections will also be stained with neutral red. In general, however, it seems that most cells having what may be a connected system of vacuoles will not show continuous neutral-red staining, thus revealing a reticular structure.

The situation may be compared to what is found in the gross digestive apparatus of the Chaetogaster worms; the pharynx shows no sign of stain; sharply at the beginning of the crop staining of the lumen begins, past the sphincter opening into the gizzard staining of the lumen ceases entirely. Yet the continuous nature of the digestive lumen is unquestioned. In an intracellular digestive system of minute dimensions where even large vacuoles are invisible unstained in the cytoplasm, it is evident that only those parts of the system into which enzyme is secreted will be made visible by neutral-red staining. This is just as true in amoeboid-like cells with discontinuous vacuoles—no connection with the outside is revealed by staining. That such connections can be revealed any more definitely during life than the indirect evidence that colloidal substances pass through them is doubtful. That such connections can be universally and satisfactorily demonstrated after death is also entirely improbable, for it is almost always upon the surfaces of the chromophile material usually surrounding the expansions of the system, or the vacuoles, that impregnating substances and osmic fumes are deposited (Marguerite Parat, '28). The canalicular system of the cell, when it is at all possible to fix such a watery and delicate apparatus, will not completely correspond, except in rare cases, to that portion of the system surrounding the vacuoles proper where the lipoidal substance may be fixed into a clear-cut reticulum.

It must also be borne in mind that the vacuolar system is subject to change. This is very obvious in Protozoa. In Daphnia it is possible to watch certain of the phagocytic cells and to identify these same cells again when the animal is transferred back and forth from the culture (individual) to the cover-slip. There are slow changes in the form and position of the vacuolar system. In these cells the changes are not marked, however, as Bensley ('11) found them in the pancreas cells where activity is periodic, whereas in Daphnia it seems to be continuous. Bensley says of the neutral-red bodies: ". . . . they wholly disappear from the pancreatic cell after a sufficient period of rest, while a few hours stimulation will cause their reappearance in the usual situation." It is well recognized that the Golgi apparatus is likewise subject to change. Many of these fluctuations have been attributed by authors to their technique, whereas it may be possible to correlate most of them with changes in neutral-red manifestations. That the chromophile substance is intimately concerned with either secretion into the vacuole or absorption from it is surely to be presumed.

Aside from changes during yolk formation corresponding to vacuolar changes (Hibbard, '28), a striking example of changes in the Golgi is described by Bowen ('26) in the inguinal gland of the rabbit. A series of droplets in a young cell is gradually merged by "the disappearance of the protoplasmic partitions and built up into a single amorphous whole." This identical process may be followed in the cells of the shell gland of the young Daphnia developing its first set of eggs. A comparison of this process revealed entirely by neutral red may be made with Bowen's figures of the process as demonstrated by a Golgi technique. It is probable that an intense Golgi substance corresponds with, not alternates with, intense hydrolytic activity.

Mitochondria generally decrease in old tissue cultures; vacuoles stainable with neutral red increase. In brief, this is the situation Lewis and Lewis ('24) summarize in their work on these cellular constituents. They call the vacuoles 'degeneration vacuoles,' in recognition of the poverty of the medium ('22). Such vacuoles increase, at the expense of the cytoplasm, until the cell is a fragile framework. According to the theories developed in this study, such vacuoles are in truth 'starvation' indicators. Constant invagination, whether vacuole formation be of the continuous or discontinuous type, is an attempt to take in all of the food possible from a weak medium. Such a slight amount of nutriment is concentrated by each vacuole that the energy invested is greater than the energy derived from the food. In addition, each vacuole calls forth enzymes, undoubtedly protein-like products derived from the complex lipoidal Golgi. In fact, excessive enzymes are probably secreted as the hunger stimulants to greater activity. Exhaustion of the Golgi may result in stimulating reserves from the mitochondria. And so the mitochondria decrease and disappear at starvation.

This is the opposite of the condition found in the highly stimulated Paramecium in a rich medium. Initially increased vacuole formation quickly results in increased mitochondria until the cell becomes crowded with them eventually: new vacuole formation ceases early and existing vacuoles are promptly excreted. Few vacuoles, but greatly increased lipoidal substance is a sign of too abundant food and may be just as pathological in the extreme as the conditions of numerous vacuoles and few lipoidal elements represent the opposite extreme.

These views do not seem to be in harmony with those of Bissachi ('16) and some others who find mitochondria increased in starvation. Consideration of the sum total of the lipoidal constituents of the cell and the relations between those concerned with hydrolysis and those related to synthesis may reconcile many observations in pathological and experimental cytology.

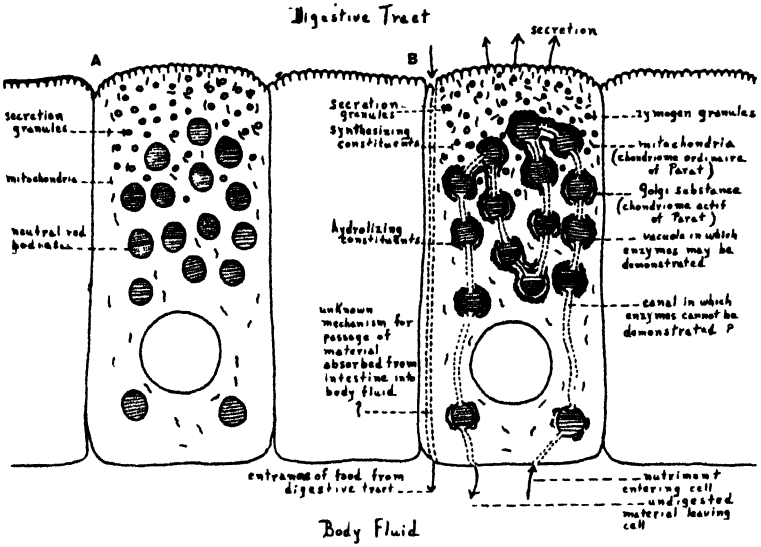

Digestive Tract
A
B
secretion
secretion granules
mitochondria
neutral red bodies
Secretion granules
synthesizing constituents
hydrolizing constituents
unknown mechanism for passage of material absorbed from intestine into body fluid
?
entrance of food from digestive tract
Body Fluid
zymogen granules
mitochondria (chondriome ordinaire of Parat)
Golgi substance (chondriome actif of Parat)
vacuole in which enzymes may be demonstrated
canal in which enzymes cannot be demonstrated ?
nutriment entering cell
undigested material leaving cell

Fig. D A secretory cell, e.g., intestinal cell. a. The cytoplasmic inclusions visible in vivo. b. Cytoplasmic relationships suggested by the neutral-red reaction.

The observer of a secreting cell such as a Daphnia digestive cell sees in the cytoplasm only some neutral-red bodies, mitochondria, and secretion granules. With the rationale of the neutral-red reaction in mind and the morphological and physiological relationships suggested with its aid, a schematic map of such a digestive cell may be formulated (fig. D).

CONCLUSIONS

In living organisms neutral red combines with enzymes wherever they exist in digestive lumina, whether these be intra- or extracellular; and also, when cellular vacuolar systems are permeable to the dye, the presence of enzymes may be demonstrated upon cytoplasmic bodies usually recognized as the mitochondria. There is thus revealed in animal cells a hydrolytic system composed of invaginated watery vacuoles and a synthetic system composed of cytoplasmic lipoidal bodies. The nature of the lipoidal Golgi structure usually intimately related to the vacuolar system, of parabasal bodies of lipoidal composition, of rhabdites much like parabasal bodies and other modifications of similar substance suggests the adaptability of but one fundamental cytoplasmic inclusion—the mitochondrial complex.

Neutral red therefore becomes, for the cytologist and the cellular physiologist, an indicator of two cellular processes, hydrolysis and synthesis, and their structures. It also reveals something of the nature of the following problems:

Neutral red may follow the course of nutriment throughout an animal organism, indicating the relationships and secretions of each system of cells. Each tissue is not only specific in its hydrolytic and synthetic complex, but it is specific in its nutriment selection as well as its secretion. The endocrine system thus comprises every tissue in the body. Of prime importance in the endocrine chain is the phagocytic, or, in vertebrates, the endo-reticular system.

Neutral red indicates that eggs developing in the ovary receive abundant nutriment, show active hydrolysis in the early stages, this giving way to very active synthesis in later stages, followed by extensive dehydration in the ripe egg, involving the giving off of superfluous enzymes through the cortex. These secretions upon the surface of the egg seem to be hydrolytically active.

Developing spermatozoa are poorly nourished. More or less limited hydrolysis gives way to dehydration and loss of all anabolic functions manifest by the coalescence of the mitochondria to form a Nebenkern and the coalescence of the vacuolar elements and their accompanying lipoidal structures to form the markedly dehydrated acrosome.

The acrosome may become permeable to water through a cytological agent, thereby activating a concentrated secretion and producing a powerful hydrolytic agent. The process of fertilization would then be the hydrolytic action of the mild secretion of the egg cortex upon an adjacent sperm head, whereupon the injured sperm digests a path for itself into the egg cytoplasm or, in better-protected eggs, down the path of the egg nutritive canaliculum. The identity of the egg and sperm secretions is manifest by neutral red.

Eggs stained with neutral red during development in the ovary lose all vacuoles and therefore all sign of neutral-red staining as they progress in yolk synthesis. The ripe egg shows no staining of the interior until development is initiated and has progressed to some definite stage. Neutral-red staining then appears, in the blastomeres in a homolecithal egg and around the yolk spheres in a centrolecithal egg. The reappearance of hydrolysis suggests the breaking down of a uniform substance for resynthesis—in other words, the beginning of embryological differentiation.

BIBLIOGRAPHY

Adams, A. E., and Fazile Shevket 1929 The normal blood picture of white rats and the changes in the picture following thyroid feeding. Physiol. Zool., vol. 2, pp. 181–220.

Adowa, A. N. 1924 Zur Frage nach den Fermenten von Utricularia vulgaris, I. Biochem. Zeitschr., Bd. 150, S. 101–107.

Ball, G. H. 1927 Studies on Paramecium. III. The effects of vital dyes on Paramecium caudatum. Biol. Bull., vol. 52, pp. 68–78.

Bayliss, W. M. 1914 The nature of enzyme action. Monographs on Biochemistry. Longmans, Green & Co., London.

——— 1923 Interfacial forces and phenomena in physiology. Methuen & Co., London.

Bechold 1919 Colloids in biology and medicine.

Becker, E. R. 1926 Vital staining and reduction of vital stains by Protozoa. Biol. Bull., vol. 50, pp. 235–238.

Bensley, R. R. 1910 On the nature of the canalicular apparatus of animal cells. Biol. Bull., vol. 19, pp. 179–194.

Bensley, R. R. 1911 Studies on the pancreas of the guinea-pig. Am. Jour. Anat., vol. 12, pp. 297–388.

——— 1929 The gastric glands. Cowdry's Special Cytology.

Bissachi, P. 1916 Del condrioma delle cellule epiteliali del villo intestinale nel digiuno prolungato e nella rialimentazione dopa inquesto. Bull. d. sc. med., Bologna, T. 9, p. 445.

Blagoveshchenski, A. 1921 The specific action of plant proteases. (In Russian.) Jour. Russian Bot. Congress, vol. 1, pp. 21–22.

Bodine, J. H. 1929 In press.

Bowen, R. H. 1926 The Golgi apparatus in gland cells. Quart. Jour. Micros. Sci., vol. 70, pp. 193–220.

——— 1926 The Golgi apparatus. Its structure and functional significance. Anat. Rec., vol. 32, pp. 151–194.

——— 1927 Golgi apparatus and vacuome. Ibid., vol. 35, pp. 309–336.

——— 1928 The methods for the demonstration of the Golgi apparatus. I. Intra-vitam observation. Vital staining. Ibid., vol. 38, pp. 293–320.

——— 1928 VI. Protozoa. The vacuome. Plant tissues. Ibid., vol. 40, pp. 225–276.

Calkins, G. N. 1926 Biology of the Protozoa. Lea & Febiger, Philadelphia.

Calkins, G. N., and Bowling, R. 1929 Studies on Dalasia frontata Stokes. II. Cytology, gametogamy and conjugation. Archiv. f. Prot., Bd. 66, S. 11–32.

Causey, D. 1926 Mitochondria in ciliates, with special reference to Paramecium caudatum, Ehr. Univ. Cal. Pub. Zool., Berkeley, vol. 28, pp. 231–250.

Chambers, R. 1928 Intracellular hydrion concentration studies. I. The relation of the environment to the pH of protoplasm and of its inclusion bodies. Biol. Bull., vol. 55, pp. 369–376.

Champy, C. 1923 La spermatogenèse chez Discoglossus pictus (Otth.). Arch. de Zool. Exp., T. 62, pp. 1–52.

Chickering, A. M. 1927 Spermatogenesis in the Belastomatidae. Jour. Morph. and Physiol., vol. 44, pp. 541–607.

Child, C. M., and Deviney, E. 1926 Physiology of Paramecium. Jour. Exp. Zoöl., vol. 43, pp. 257–312.

Child, H. J. 1926 Studies on the ingestion of leucocytes and on mitosis in Endamoeba gingivalis. Univ. Calif. Pub. Zool., vol. 28, pp. 251–284.

Chlopin, Nikolaus G. 1927 Experimentelle Untersuchungen über die sekretorischen Progresse im Zytoplasma. I. Über die Reaktion der Gewebselemente auf intravitale Neutralrotfärbung. Arch. exp. Zellforsch., Bd. 4, S. 462–599.

Claus, C. 1876 Zur Kenntniss der Organisation und des feineren Baues der Daphniden und verwandter Cladoceren. Zeit. f. wiss. Zool., Bd. 27, S. 362–402.

Cohn, E. J. 1917 The relation between the hydrogen ion concentration of sperm suspensions and their fertilizing power. Anat. Rec., vol. 11, p. 530.

Conklin, E. G. 1924 Cellular differentiation. Cowdry, General Cytology.

CONN, H. J. 1925 Biological stains. Geneva, N. Y.

COVELL, W. P. 1927 A quantitative study of the Golgi apparatus in spinal ganglion cells. Anat. Rec., vol. 35, pp. 149–157.

———— 1928 A microscopic study of pancreatic secretion in the living animal. Anat. Rec., vol. 40, pp. 213–224.

COVELL, W. R., AND SCOTT, G. H. 1928 An experimental study of the relation between granules stainable with neutral red and the Golgi apparatus in nerve cells. Anat. Rec., vol. 38, pp. 377–400.

COWDRY, E. V. 1924 General cytology. University of Chicago Press.

———— 1928 Special cytology. Paul B. Hoeber, New York.

DAWSON, A. B. 1928 The 'segregation apparatus' of the amphibian erythrocyte and its possible relation to the Golgi apparatus. Anat. Rec., vol. 39, pp. 137–154.

DUESBERG, J. 1926 Discussion au sujet de l'appareil de Golgi. Compt. Rend. de l'Ass. des Anat., Liège, 21 Réunion, p. 613.

DOCHEZ 1909 Jour. Exper. Med., vol. 11, p. 718.

EHRLICH, P. 1894 Vortrag über das Neutralrot. Verein f. innere Medizin z. Berlin. München. med. Wochenschr.

EPSTEIN, A. A., AND ROSENTHAL, N. 1924 Trypsin-insulin reaction and causation of diabetes. Amer. Jour. Physiol., vol. 71, pp. 316–338.

FAURÉ-FREMIET, E. 1909 Vacuoles colorables par le rouge neutre chez un infusoire. Compt. Rend. de l'Ass. des Anat., T. 11.

———— 1910 Sur la valeur des indications microchimiques fournies par quelques colorants vitaux. Anat. Anz., Bd. 40, S. 378–380.

———— 1910 Étude sur les mitochondries des protozoaires, et des cellules sexuelles. Arch. d'Anat. micr., T. 2, pp. 457–648.

GARDNER, L. V. 1927 Preserving supravital staining with neutral red in paraffin sections of the lung. Proc. Soc. Exp. Biol. and Med., vol. 24, pp. 646–648.

GATENBY, J. B. 1919 V. Limnaea. The cytoplasmic inclusions of germ cells. Quart. Jour. Micros. Sci., vol. 63, pp. 445–492.

———— 1920 On the relationship between the formation of yolk and mitochondria and Golgi apparatus during oogenesis. Jour. Roy. Micros. Soc., vol. 151, pp. 129–156.

GIES, W. S. 1901 On spermatozoa contain an enzyme having the power of causing the development of mature ova? Amer. Jour. Physiol., vol. 6, pp. 53–76.

GLASER, O. 1913 Note on the synthesis of ethyl butrate in egg secretions. Science, vol. 55, p. 486.

———— 1921 Note on the pigment of Arbacia egg-secretion. Biol. Bull., vol. 41, pp. 256–258.

———— 1921 Duality of egg secretion. Am. Nat., vol. 55, pp. 368–373.

———— 1922 The hydrolysis of higher fats in egg-secretion. Biol. Bull., vol. 43, pp. 175–183.

———— 1923 Copper, enzymes and fertilization. Ibid., vol. 44, pp. 79–104.

GLICKLHORN, J. LE, UND R. KELLER 1926 Organspezifische Differenzierung des Tierkörpers durch elektive Vitalfärbungen. Ein Beitrag zum Spezifitätsproblem in der Biologie. Biol. Generalis, Bd. 2, S. 537–564.

GOODRICH, H. P. 1928 Reactions of Gammarus to injury and disease, with notes on some microsporidial and fungoid diseases. Quart. J. Micr. Sc., vol. 72, pp. 325–353.

GREENWOOD, M. 1894 On the constitution and the mode of formation of 'food vacuoles' in Infusoria, etc. Philos. Trans. Roy. Soc. London, p. 353.

GRASSE-ALLERMANN, W. 1909 Amoeba terricola. Archiv. f. Prot., Bd. 17, S. 203–257.

HARVEY, E. N. 1911 Studies on the permeability of cells. Jour. Exp. Zoöl., vol. 10, pp. 507–556.

HIBBARD, HOPE 1928 Contribution à l'étude de l'ovogenèse, de la fécondation, et le l'histogenèse chez Discoglossus pictus Otth. Arch. de Biol., T. 38, pp. 251–325.

———— 1928 Cytoplasmic constituents in the developing egg of Discoglossus pictus Otth. Jour. Morph. and Physiol., vol. 45, pp. 233–258.

HILL, A. G., AND GATENBY, J. B. 1927 The oogenesis of Daphnia by intra vitam and post vitam staining. Nature, London, vol. 120, p. 917.

HOGBEN, L. T. 1924 The pigmentary effector system. A review of the physiology of colour response. Oliver & Boyd, London.

———— 1927 The comparative physiology of internal secretion. Cambridge, at the University Press.

HOLZBERG, H. L. 1913 A new method of isolating trypsin. Jour. Biol. Chem., vol. 14, pp. 335–339.

HORNING, E. S. 1926 Observations on mitochondria. Austral. Jour. Exp. Biol. and Med. Sci., vol. 3, pp. 149–160.

———— 1927 Mitochondrial behavior during the life cycle of Nyctotherus cordiformis. Austral. Jour. Exp. Biol. and Med. Sci., vol. 4, pp. 69–73.

HORNING, E. S., AND PETRIE, A. H. K. 1927 The enzymatic function of mitochondria in the germination of cereals. J. Roy. Soc., Ser. B, p. 102.

HSÜ, TSON-HIA 1924 Über die Absorption des Trypsins durch Filtrierpapier. Biochem. Zeitschr., Bd. 144, S. 303–307.

HUGOUNENQ, L. 1927 Nature des diastases et mécanisme de leurs actions avec quelques applications au problème de l'héridité. Rev. Gen. Sci. Pures and Appl., T. 38, pp. 70–76.

HUGOUNENQ, L., ET J. LOISELEUR 1925 Sur la constitution des diastases protéolytiques et le mécanisme de leur action. Bull. Soc. Chim. Biol., T. 7, pp. 955–973.

JOCHMANN 1908 Ztschr. f. Hyg., Bd. 61, S. 71.

JOYET-LAVERGNE, P. 1927 Sur le rôle du chondriome dans le metabolisme cellulaire. Compt. rend. Soc. Biol., T. 97, pp. 327–330.

JUST, E. E. 1928 Initiation of development in Arbacia. V. The effect of slowly evaporating sea-water and its significance for the theory of auto-pathenogenesis. Biol. Bull., vol. 55, pp. 358–369.

———— 1928 Hydration and dehydration in the living cell. I. The effect of extreme hypotony on the egg of Nereis. Physiol. Zoöl., vol. 1, pp. 122–135.

KATER, J. MCA. 1928 Morphological aspects of protoplasmic and deutoplasmic synthesis in oogenesis of Cambarus. Zeit. f. Zellforsch. u. mikros. Anat., Bd. 8, S. 186–221.

KIESEL, ALEXANDER 1924 Études sur la nutrition de l'Utricularia vulgaris. Ann. Inst. Pasteur, T. 38, pp. 859–891.

KING, S. D. 1926 Cytology of Anoplophyra. Quart. Jour. Micros. Sci., vol. 70, pp. 693–700.

——— 1927 A review. The Golgi apparatus of Protozoa. Jour. Roy. Micros. Sci., vol. 47, pp. 342–355.

KING, S. D., AND GATENBY, J. B. 1926 Note on certain new bodies in Opalina renarum, presumed to represent the Golgi elements. Quart. Jour. Micros. Sci., vol. 70, pp. 217–220.

KOEHRING, V. 1925 The spermatheca of Eurycea bislineata. Biol. Bull., vol. 49, pp. 250–264.

KOFOID, C., AND SWEZY, O. 1926 The cytology of Endamoeba gingivalis. Univ. Calif. Publ. Zool., vol. 20, pp. 169–198.

KORSAKOVA, MARIE 1910 Ueber den Einfluss der Zelllipoide auf die Autolyse der Weizenkeime. Biochem. Zeitsch., Bd. 28, S. 121–126.

LANDIS, E. M. 1925 Conjugation in Paramecium. Jour. Morph. and Physiol., vol. 40, pp. 11–167.

LEWIS, M. R. 1917 The effect of certain vital stains upon the development of the eggs of Cerebratulus lacteus, Echinorachnius parma and Lophius piscatorius. Anat. Rec., vol. 13, pp. 21–36.

LEWIS, W. H. 1919 Degeneration granules and vacuoles in the fibroblasts of chick embryos cultivated in vitro. Johns Hopkins Hosp. Bull., vol. 30, pp. 81–90.

LEWIS, W. H., AND LEWIS, M. R. 1924 Behavior of cells in tissue cultures. Cowdry's General Cytology.

LEWIS, M. R., AND LEWIS, W. H. 1926 Transformation of mononuclear blood cells into macrophages, epithelioid cells, and giant cells in hanging drop blood cultures from lower vertebrates. Contrib. to Embr., no. 96, Carnegie Pub., 363.

LEWIS, M. R., AND ROBERTSON, WM. R. 1916 The mitochondria and other structures observed by the tissue culture method in the male germ cells of Chorthippus. Biol. Bull., vol. 30, pp. 99–124.

LILLIE, F. R. 1913 Studies of fertilization. V. The behavior of the spermatozoa of Neries and Arbacia, with special reference to egg extractives. Jour. Exp. Zoöl., vol. 14, pp. 515–574.

——— 1914 Studies of fertilization. VI. The mechanism of fertilization is Arbacia. Jour. Exp. Zoöl., vol. 16, pp. 523–590.

——— 1915 Sperm agglutination and fertilization. Biol. Bull., vol. 28, pp. 18–33.

——— 1915 Studies on fertilization. VII. Analysis of variations in the fertilizing power of sperm suspensions of Arbacia. Ibid., vol. 28, pp. 229–251.

——— 1919 Problems of fertilization. Univ. of Chicago Press, Chicago.

LILLIE, F. R., AND JUST, E. E. 1924 Fertilization. Cowdry's General Cytology.

LILLIE, R. S. 1924 Reactivity of the cell. Cowdry's General Cytology.

LOEB, J. 1914 Cluster formation of spermatozoa caused by specific substance from eggs. Jour. Exp. Zoöl., vol. 17, pp. 123–140.

——— 1916 The organism as a whole from a physico-chemical viewpoint. New York: G. P. Putnam's Sons.

MA, WEN-CHAO 1928 The relation of mitochondria and other cytoplasmic constituents to the formation of secretion granules. Am. Jour. Anat., vol. 41, pp. 51–63.

MACKLIN, C., AND MACKLIN, M. 1928 The intestinal epithelium. Cowdry's Special Cytology.

MAIER, H. N. 1903 Der feinere Bau der Wimperapparate der Infusorien. Arch. f. Prot., Bd. 2, S. 73–179.

MARSTON, H. R. 1923 Compounds of proteolytic enzymes with dyes. Biochem. Jour., vol. 17, pp. 851–859.

——— 1926 On the acceleration of enzymatic synthesis of proteins by lipoidal emulsions. Austral. Jour. Exp. Biol. and Med Sci., vol. 3 (4), pp. 233–236.

MAST, S. O. 1926 Structure and locomotion in Amoeba. Jour. Morph. and Physiol., vol. 41, pp. 347–426.

MAXIMOW, A. 1928 The macrophages or histiocytes. Cowdry's Special Cytology.

METSCHNIKOFF, E. 1905 Immunity in infective diseases. Cambridge Univ. Press.

MEYER, J. DE 1911 Observations et expériences relatives à l'action exercée par des extracts d'oeufs et d'autres substances sur les spermatozoides. Arch. de Biol., T. 26, pp. 65–101.

MINCHIN, E. A. 1917 An introduction to the study of Protozoa. London: Arnold.

MONNÉ, L. 1927 Observations sur les spermatocytes des mollusques après coloration vitale (appareil de Golgi, vacuome). Compt. rend. Soc. Biol., T. 97, pp. 1450–1452.

MOUTON, H. 1902 Recherches sur la digestion chez les Amibes et sur leur diastase intracellular. Thèse prés. 1902. Paris.

MURRAY, M. R. 1926 Secretion in the amitotic cells of the cricket egg follicle. Biol. Bull., vol. 50, pp. 210–234.

NATH, VISHWA 1925 Mitochondria and sperm-tail formation with particular reference to moths, scorpians, and centipedes. Quart. Jour. Micr. Sci., vol. 69, pp. 643–659.

——— 1926 On the present position of the mitochondria and the Golgi apparatus. Biol. Rev., vol. 2, pp. 52–79.

——— 1928 Studies in the origin of yolk. I. Oogenesis of the spider, Crossopriza lyoni. Quart. Jour. Micr. Sci., vol. 72, pp. 277–300.

NATH, VISHWA, AND MEAN T. HASAIN 1929 Oogenesis of Scopopendra. Quart. Jour. Micr. Sci., vol. 72, pp. 403–418.

NIRENSTEIN, E. 1905 Ernährungsphysiologie der Protisten. Zeit. f. allg. Physiol., Bd. 5, S. 434.

OPIE, E. L. 1909–1910 Inflammation. Harvey Lectures. J. B. Lippincott, Philadelphia.

OWENS, H. B., AND R. R. BENSLEY 1929 On osmic acid as a microchemical reagent, with special reference to the reticular apparatus of Golgi. Am. Jour. Anat., vol. 44, pp. 79–110.

PALLADIN, VLADIMIR I. 1926 Plant physiology. Blakiston's Son & Co., Philadelphia.

PAPPENHEIMER 1916 The Golgi apparatus. Anat. Rec., vol. 11, pp. 107–148.

PARAT, MARGUERITE 1928 Vacuome et appareil de Golgi de la glande pelvienne du Triton marbre au course de l'évolution glandulaire. Compt. rend. Soc. Biol., T. 94, pp. 201–203.

PARAT, M. 1925 Vacuome et appareil de Golgi. Compt. rend. Ass. des Anat., Turin, p. 306.

——— 1926 Les constituants cytoplasmiques de la cellule génitale femelle, l'ovocyte de Ciono intestinale L. Compt. rend. Soc. Biol., T. 94, pp. 444–446.

——— 1927 A review of recent developments in histochemistry. Biol. Rev., vol. 2, p. 285.

——— 1928 Rapports du chondriome et du vacuome dans la 'zone de Golgi'; constitution de cette zone. Extrait des Compt. rend. de l'Ass. des Anat., Prague.

PARAT, M., AND PAINLEVÉ 1924 Appareil réticulaire interne de Golgi, trophosponge de Holmgren et vacuome. Compt. rend. Ass. Sci., T. 179, p. 844.

——— 1926 L'appareil de Golgi des cellules génitales mâles d'Helix et des autres Pulmonés. Compt. rend. Soc. Biol., T. 94, p. 745.

PEACOCK, A. D., AND GRESSON, R. A. 1928 The rôles of the nurse cells, oocytes and follicle cells in Tenthridinid oogenesis. Quart. Jour Micr. Sci., vol. 71, pp. 541–562.

POISSON, R. 1927 Recherches sur quelques processus observés dans les éléments sexuels jeunes de Notonecta maculata Fab. Arch. de Zool. Exp., T. 66, pp. 23–70.

POPA, G. T. 1927 A lipo-gel reaction exerted by follicular fluid upon spermatozoa and its significance (Lillie's reaction). Biol. Bull., vol. 52, pp. pp. 223–237.

——— 1927 The distribution of substances in the spermatozoon (Arbacia and Neries) studied by intra-vitam stains and by stains of lipoids according to the method of Schumacher. Biol. Bull., vol. 52, pp. 238–257.

PRENANT, A. 1913 L'origine mitochondriale des pigments. Compt. rend. Soc. Biol., T. 74, pp. 926–929.

PROWAZEK, S. 1897 Vitalfärbungen mit Neutralrot an Protozoa. Zeitschr. f. wiss. Zool., Bd. 63, S. 187.

RÉNYI, G. S. 1924 Studies on pigment genesis. The nature of so-called 'Pigmentbildner.' Jour. Morph. and Physiol., vol. 39, pp. 415–437.

RICHARDS, A., AND WOODWARD, A. E. 1915 Notes on the effect of X radiation on fertilization. Biol. Bull., vol. 28, pp. 140–147.

ROBERTSON, T. B. 1907 Studies in the chemistry of the ion-proteid compounds. Jour. Biol. Chem., vol. 2, pp. 317–384.

——— 1912 On the extraction of a substance from the sperm of a sea urchin (Strongylocentratus p.) which will fertilize the eggs of that species. Jour. Biol. Chem., vol. 12, pp. 1–11.

——— 1928 The combination of proteins with acids and bases, with some observations on the origin of viscosity in protein solutions. Colloid Chemistry, J. Alexander. Chem. Cat. Co., New York.

SABIN, F. R. 1921 Studies on blood. The vitally stainable granules as a specific criterion for erythroblasts and the differentiation of the three strains of white blood cells as seen in the living chick's yolk-sac. Johns Hopkins Hos. Bull., vol. 32, pp. 314–321.

SABIN, F. R., DOAN, C. A., AND CUNNINGHAM, R. S. 1925 The discrimination of two types of phagocytic cells in the connective tissues by the supravital technique. Contr. to Embr., vol. 16, Pub. Car. Inst. Wash., 361.

SAGUCHI 1917 Studies on ciliated cells. Jour. Morph., vol. 29, pp. 217–279.

SAMPSON, M. M. 1926 The parthenogenetic effect of sperm filtrates, concentrated sperm suspensions and serum of chitons on the ova of the sea urchin, Strongylocentratus f. Biol. Bull., vol. 50, pp. 202–206.

——— 1926 Sperm filtrates and dialyzates. Biol. Bull., vol. 50, pp. 301–338.

SCHAEFFER, A. A. 1916 On the feeding habits of Amoeba. Jour. Exp. Zoöl., vol. 20, pp. 529–584.

SHAPIRO, N. N. 1927 The cycle of hydrogen-ion concentration in the food vacuoles of Paramecium, Vorticella and Stylonichia. Trans. Amer. Micros. Soc., vol. 46, pp. 45–53.

SHIPLEY, P. G. 1919 Reactions of tissue cells to vital dyes. Am. Jour. Physiol., vol. 49, pp. 284–301.

SMITH, H. W., AND CLOWES, G. H. A. 1924 A further examination of the influence of hydrogen ion concentration on the physiological properties of marine eggs and sperm. Biol. Bull., vol. 48, pp. 323–344.

STRANGEWAYS, T. S. P., AND CANTI, R. G. 1927 The living cell in vitro as shown by dark-ground illumination and the changes induced in such cells by fixing reagents. Quart. Jour. Micr. Sci., vol. 71, pp. 1–14.

SUNDQUIST, LEONA 1925 Some enzymatic actions of Nereocystis luetkeana. Publ. Puget Sound Biol. Sta., 'vol. 3, pp. 331–336.

SVETLON, P. 1929 Archiv. f. Entwick. d. Org., Bd. 114, S. 771.

VOÏNOV, D. 1927 Lè vacuome et l'appareil de Golgi dans les cellules genitales mâles de Notonecta glauca L. Arch. Zool. Exp., T. 67, pp. 1—22.

——— 1928 La pigmentogenèse chez les larves de Simulium. Arch. de Zool. Exp., T. 67, pp. 223–258.

WALLICH, G. C. 1863 Further observations on an undescribed indigenous Amoeba. Am. Mag. Nat. Hist., ser. 3, vol. 11, pp. 365–371.

WEISMAN, A. 1876–1879 Beiträge zur Naturgeschichte der Daphnoiden. Leipzig.

WENRICH, G. H. 1928 Eight well-defined species of Paramecium. Trans. Am. Micros. Soc., vol. 47, pp. 275.

WENYON, C. M. 1926 Protozoology. Wood, New York.

WILLIER, B. H., HYMAN, L. H., RIFENBURGH, S. A. 1925 A histochemical study of intracellular digestion in Triclad flatworms. Jour. Morph. and Physiol., vol. 40, pp. 299–340.

WILSON, C. N. 1926 A comparative study of several samples of neutral red and their effects on Paramecium caudatum. Stain Technology, vol. 1-2, pp. 115–123.

WINKELSTEIN, A., AND MARCUS, J. M. 1929 Excretion of neutral red in the stomach in achylia gastrica. Jour. Am. Med. Ass., vol. 92, pp. 1238–1240.

WISLOCKI, G. B. 1916 The staining of amphibian larvae with benzidine dyes with especial reference to the behavior of the lymphatic endothelium. Am. Jour. Physiol., vol. 42, pp. 124–132.

WOODWARD, A. 1918 Studies on the physiological significance of certain precipitates from the egg secretions of Arbacia and Asterias. Jour. Exp. Zoöl., vol. 26, pp. 459–501.

EXPLANATION OF PLATES

All figures are of in-vivo material unless otherwise stated. All figures of plates 1 and 4 are camera-lucida drawings, as are the figures of plate 2, with the exception of figures 41, 46, 47, 54, and 55. The figures are all somewhat diagrammatic in that details not directly concerned with the neutral-red reaction have been omitted.

PLATE 1

EXPLANATION OF FIGURES

1 Paramecium in a 1: 10,000,000 solution of neutral red after ten to fifteen minutes.

2 Paramecium in a 1: 10,000,000 solution of neutral red after two hours. Note increased number of vacuoles.

3 Paramecium in a 1: 10,000,000 solution of neutral red after twenty-four hours. Note vacuoles only in the posterior region. After about forty-eight hours, however, the vacuoles are distributed as in figure 1 and are slightly more numerous than in unstained organisms.

4 Paramecium in a 1: 2,000,000 solution of neutral red after twenty-four hours. Note the staining of cytoplasmic granules and the number and distribution of vacuoles.

5 Paramecium in a neutral-red dilution less than 1: 1,000,000, showing the staining of the cytoplasmic granules and the parabasal bodies. Food vacuoles are few and watery. One vacuole is opening into the cytophyge.

6 Paramecium in a 1: 2,000,000 solution of neutral red after two hours. Excretion of vacuoles is rapid. Two vacuoles are shown coalescing near the cytophyge. One vacuole, emptying, shows the instantaneous loss of color from the food particles upon reaching the exterior.

7 Paramecium during rapid evacuation in the urea-urease reaction. The extreme vacuolization of the cytoplasm is shown and also the vacuoles around the crystals. Food vacuoles coalescing before emptying.

8 Paramecium from a continuously stained culture in division. Unequal distribution of the food vacuoles seems to be common.

(Continued on p. 130)

9 Paramecia from a continuously stained culture in conjugation. No food vacuoles are present.

10 Amoeba proteus in a 1: 5,000,000 neutral-red solution after forty-eight hours. A stained rotifer is being ingested. Note that the food vacuoles are not always round. The faint color of the vacuolar fluid is not shown.

11 Amoeba proteus in a 1: 1,000,000 neutral-red solution after twenty-four hours. Staining of the cytoplasmic granules and around the crystals is shown. A recently ingested and uninjured Arcella is in one lobe.

12 Amoeba proteus in a 1: 2,000,000 neutral-red solution after four days. Divisions are numerous.

13 Amoeba verrucosa in a 1: 5,000,000 neutral-red solution from 1/23/29.

14 Amoeba verrucosa in a 1: 1,000,000 neutral-red solution after twenty-four hours, showing staining of cytoplasmic granules.

15 Amoeba limax in a 1: 2,000,000 dilution of neutral red from 2/25–3/1. The cytoplasmic granules are purple.

16 Amoeba dubia in a 1: 2,000,000 dilution of neutral red from 2/25–3/1.

17 Vorticella in a 1: 5,000,000 solution of neutral red after twenty-four hours.

18 Vorticella in a 1: 2,000,000 solution of neutral red after twenty-four hours.

19 Vorticella in a neutral-red solution less than 1: 1,000,000, showing arrangement of staining bodies in the pellicle, several clumps of these bodies forming blotches of color and also staining bodies in the stalk.

20 Stentor (transparent) in a 1: 5,000,000 solution, 2/23–27.

21 Notosolenus in a 1: 2,000,000 solution after five days.

22 Amoeba polypodia in a 1: 1,000,000 solution after twenty-four hours. The cytoplasmic granules are magenta.

23 Mastigamoeba after twenty-four hours in a 1: 2,000,000 solution.

24 Unidentified form after one week in a 1: 10,000,000 dilution.

25 Trachelomonas after one week in a 1: 2,000,000 dilution.

26 Amoeba radiosa after one week in a 1: 2,000,000 dilution.

27 Unidentified form after one week in a 1: 2,000,000 dilution.

28 Nuclearia simplex after one week in a 1: 2,000,000 dilution.

29 Amoeba guttula after one week in a 1: 2,000,000 dilution.

30 Hyalosphenia after one week in a 1: 2,000,000 dilution.

31 Chilomonas after one week in a 1: 2,000,000 dilution.

32 Histrio after twenty-four hours in a 1: 1,000,000 solution. Stained bodies on vacuole seem to be adherent to membrane.

33 Arcella after twenty-four hours in a 1: 1,000,000 solution.

34 Stylonychia after forty-eight hours in a 1: 1,000,000 solution. Many stained bodies adherent to vacuoles.

35 Acineta after twenty-four hours in 1: 2,000,000 solution.

36 Spirostomum in a dilution less than 1: 1,000,000. The cytoplasmic granules have not been stained even when the animal was killed by too much neutral red.

37 Colpoda after twenty-four hours in 1: 1,000,000 solution.

38 Actinophrys after twenty-four hours in a 1: 2000 solution. Cytoplasmic bodies a deep purple.

39 Trichodina on tadpole tail, showing elongated food vacuoles. 1: 1,000,000 neutral red.

40 Euglena after twenty-four hours in 1: 1,000,000 neutral red. Only green chloroplasts and neutral-red bodies visible.

NEUTRAL-RED REACTION
VERA KOEHRING

PLATE 1

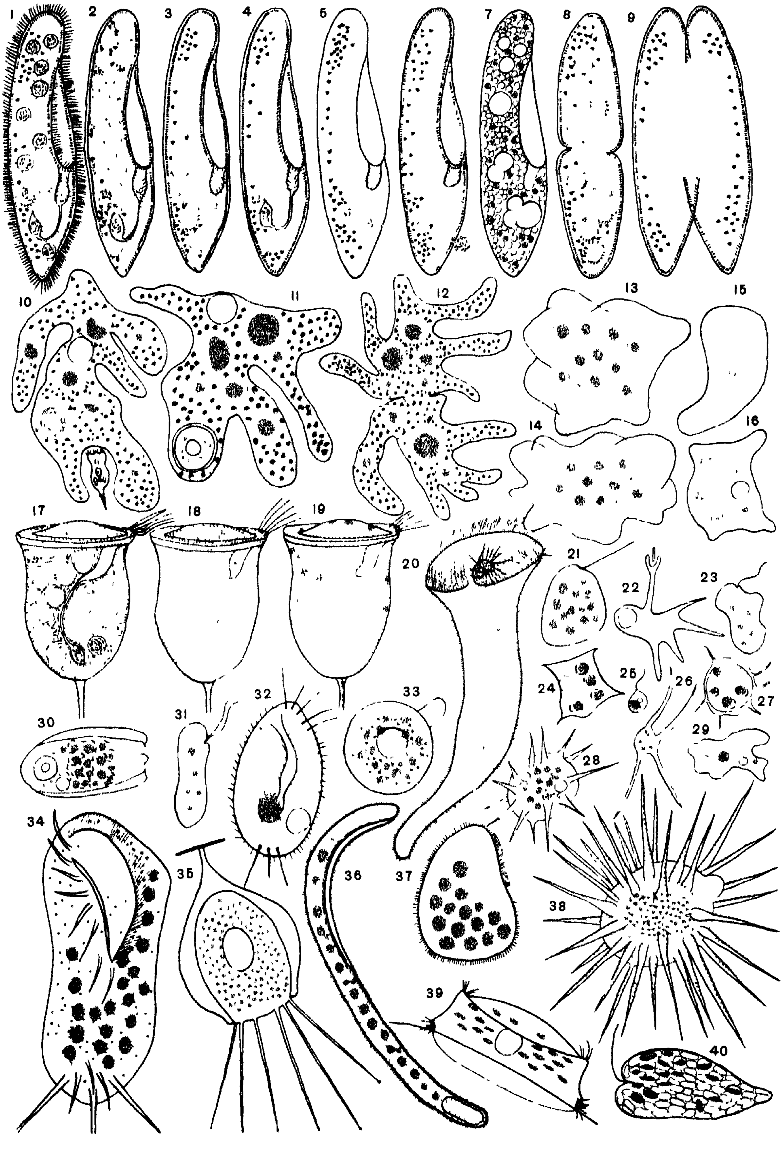


PLATE 2

EXPLANATION OF FIGURES

41 Hydra after two hours in 1:1,000,000 neutral red. Cells shown in very diagrammatic form.

42 Nematocysts from near base of body after five hours in 1:1,000,000 neutral red.

43 Nematocyst cell from tentacle after five hours in 1:1,000,000.

44 Entoderm cells from body after two hours. Mesodermal elements between do not stain.

45 Portion of tentacle after three hours. Circulating cells in the lumen are large ciliated cells and smaller cells behaving as flagellates do. 1:1,000,000 neutral red.

46 Planaria in 1:1,000,000 neutral red from 3/27–30, showing developing eggs.

47 Stenostomum after two weeks in a 1:5,000,000 solution.

48 Chaetogaster langi, showing part of pharynx, crop, gizzard, and a few cells of the intestine one-half hour after staining in 1:1,000,000 neutral red. The Arcella in the gizzard was ingested after staining and was brilliantly colored while it was in the crop. Note that no cells of the body are stained other than the gizzard cells. The solid core of clear bright pink in the crop is represented by cross hatching.

49 Head of Chaetogaster langi after five hours in 1:1,000,000 neutral red. Brain and muscles are not stained.

50 Chaetogaster langi, part of intestine and part of gizzard after five hours in 1:1,000,000 neutral red. Note in intestine five gizzard cells beginning autodigestion.

51 Part of crop and pharynx five hours after staining.

52 A gizzard cell near the end of the intestine undergoing final autodigestion. Upon disintegration, the colorless neutral-red bodies are excreted.

53 Body wall and adjacent cells to ovary in rotifer. Three oocytes. Note flame cell. After twenty-four hours in 1:1,000,000 neutral red.

54 Newly hatched snail, Physa, in 1:1,000,000 neutral red. Hepatic region is densely stained. Heart is not stained. Foot and mantle cells have delicate neutral-red bodies.

55 Gastrotrich in 1:1,000,000 neutral red.

NEUTRAL RED REACTION
VERA KOEHRING

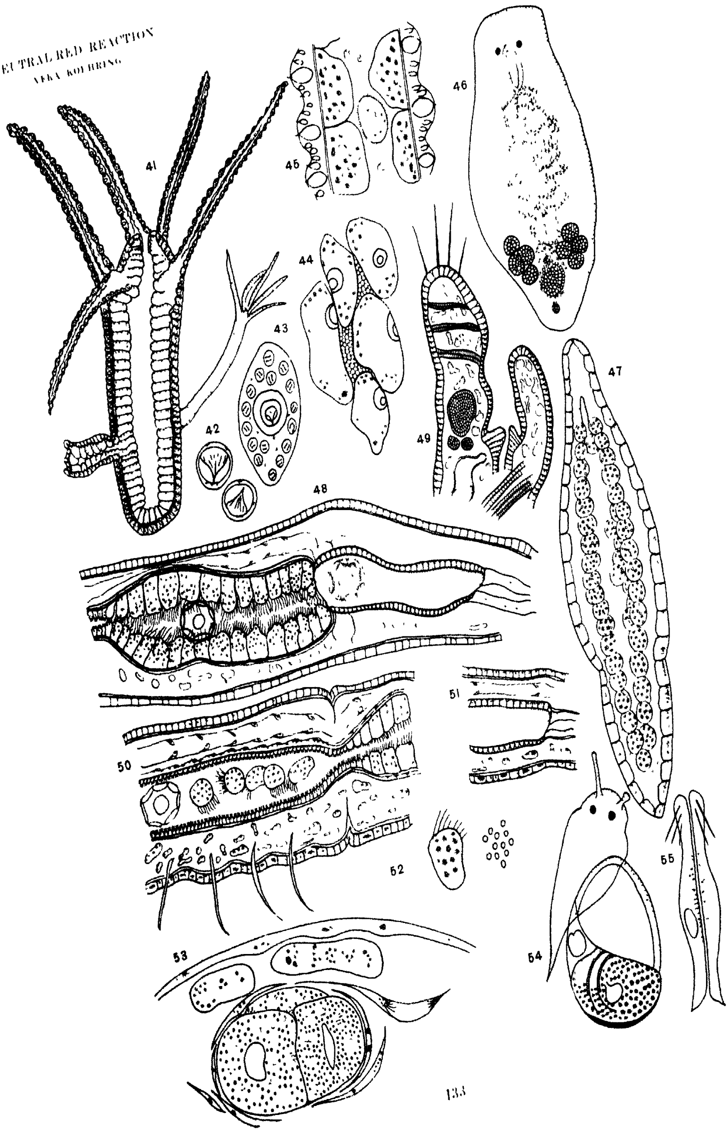


PLATE 3

EXPLANATION OF FIGURES

56 Daphnia after one hour in 1: 2,000,000 neutral red. The short oesophagus is not stained. The lumina of the foregut and the hepatic caeca are a light pink, forming a smooth core of color. No cells are stained.

57 Daphnia after two hours in 1: 2,000,000 solution. The cells of the neutral phagocytic system are beginning to stain.

58 Daphnia after three hours in 1 · 2,000,000 solution. The neutral phagocytic cells are more brilliant and the alkaline phagocytes show their neutral-red bodies. (Cannot be shown in diagram.) The ovary shows staining in the young oocytes.

59 Daphnia after twenty four hours in 1: 2,000,000 neutral red. The cells of the digestive tract, the muscles, the respiratory epithelium, the carapace cells, and the brain and sensory organs show staining so that every somatic cell in the body is stained. Some of the older eggs show no staining and developing eggs in the brood pouch are not stained. No staining in the coelomic cavity.

60 A Daphnia after forty-eight hours in 1 · 2,000,000 neutral red. There were no embryos in the brood pouch at the beginning of staining. Ripe eggs have developed during the staining period. After yolk formation they show no staining. After passing into the brood chamber, one at a time at approximately three-hour intervals, and after at least one layer of superficial cells has cleaved, the neutral red in the eggs manifests itself in staining of the yolk spheres.

61 Daphnia after five days in 1: 2,000,000 neutral red. Only the phagocytic systems and gonads are stained. The gut is lightly stained, the posterior gut at least showing the effect of a cellular secretion containing neutral red. One young individual is left in the brood pouch, fully formed and active.

62 The rotifer, Brachionus, after one hour in 1: 1,000,000 neutral red. A constriction in some forms indicates a division of stomach and intestine, the cells of both of which are ciliated amoeboid phagocytes staining brilliantly. The lumen is not stained.

63 Brachionus after twenty-four hours in 1: 1,000,000 neutral red. Every cell in the body is stained. No part of the digestive lumen or coelomic cavity is stained. The developing embryo does not stain.

64 Daphnia egg developed during staining period; after three hours in brood pouch.

65 Brachionus egg developed during staining period; at morula or early blastula stage.

PLATE 3

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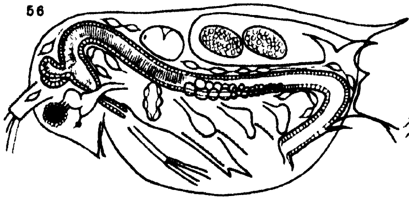

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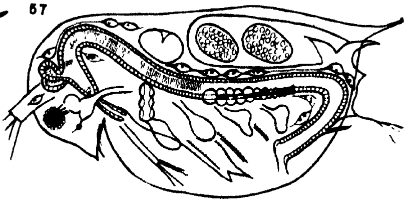

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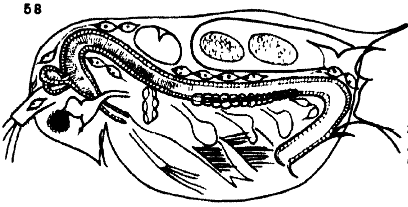

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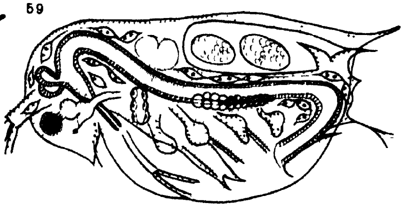

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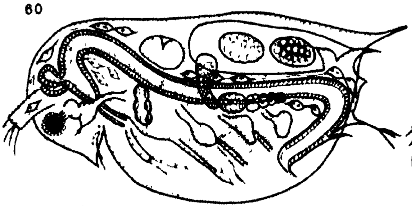

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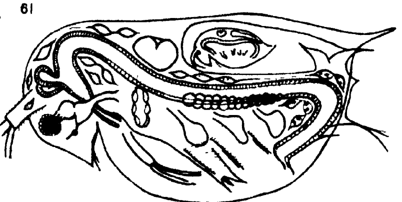

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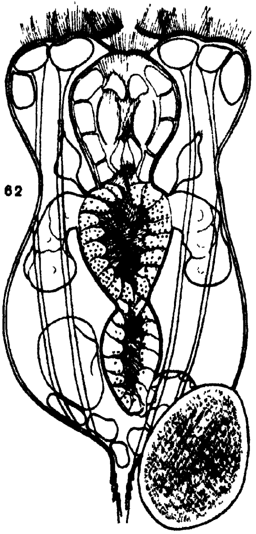

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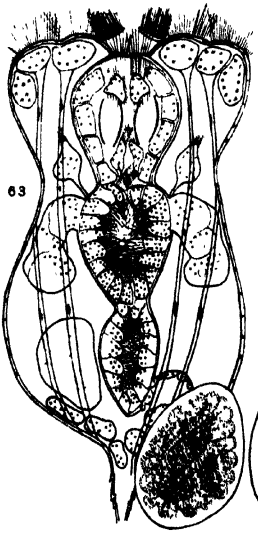

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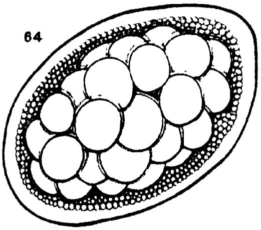

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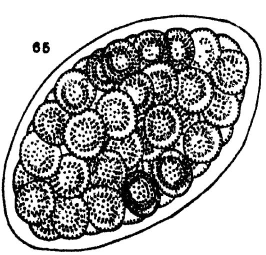


PLATE 4

EXPLANATION OF FIGURES

66 Alkaline phagocytic cell from Daphnia stained twenty-four hours in 1: 2,000,000 neutral red. Neutral red staining orange and yellow shades. Blood corpuscles adherent. Drawn at 3.00 P.M.

67 Same cell drawn at 5.00 P.M. Crescent-shaped bodies are in some cases remnants of old vacuoles no longer completely visible as staining ceases, and in other cases they are the staining portions of newly formed vacuoles. No amoeboid changes in the shapes of these cells are ever noted.

68 Stained carapace cells of female Daphnia.

69 Stained carapace cells of male Daphnia.

70 Neutral phagocyte cells from young Daphnia. Note fat-inclusions.

71 Neutral phagocyte cells from Daphnia with four embryos in brood pouch.

72 Neutral phagocytes from senile Daphnia.

73 Neutral phagocyte after seventy-two hours' staining in 1: 1,000,000 neutral red.

74 Neutral phagocytes in Daphnia with yeast disease. Two blood corpuscles and two yeast cells shown.

75 End of shell gland in young individual developing first set of oocytes. Note connections between minute neutral-red bodies.

76 Same region in shell gland in Daphnia with embryos in brood pouch and another set of eggs nearing maturity.

77 Brain and ocellus in Daphnia at height of staining.

78 Human monocyte in sealed film twenty four hours old (technique of Sabin). Narrow black rim on neutral-red bodies.

79 Developing muscle cell in head of Culex larva.

80 Later stages in muscle development in head of Culex larva.

81 Epidermis, muscles, and blood corpuscle in antenna of male Daphnia.

82 Muscle from appendage of female Daphnia. Striations not shown. Note characteristic arrangement of neutral-red bodies.

83 Part of respiratory appendage in Daphnia, showing epithelium, muscles, neutral phagocytes, and blood corpuscles at height of staining.

84 Stained supporting cell and unstained germ cell in testis of male Daphnia. No details can be distinguished in vivo.

85 Daphnia testis. No stained germ cells can be distinguished.

86 Daphnia, young oocytes.

87 Characteristic group of four cells, from Daphnia ovary, which will form one mature egg. Also adjacent Eierbehalter. Figures 86 and 87 are drawn at the same magnification (camera lucida); note increase in size of nuclei.

88 Grasshopper pericardial cell in vitro. Note two stages of neutral-red bodies. Binucleate.

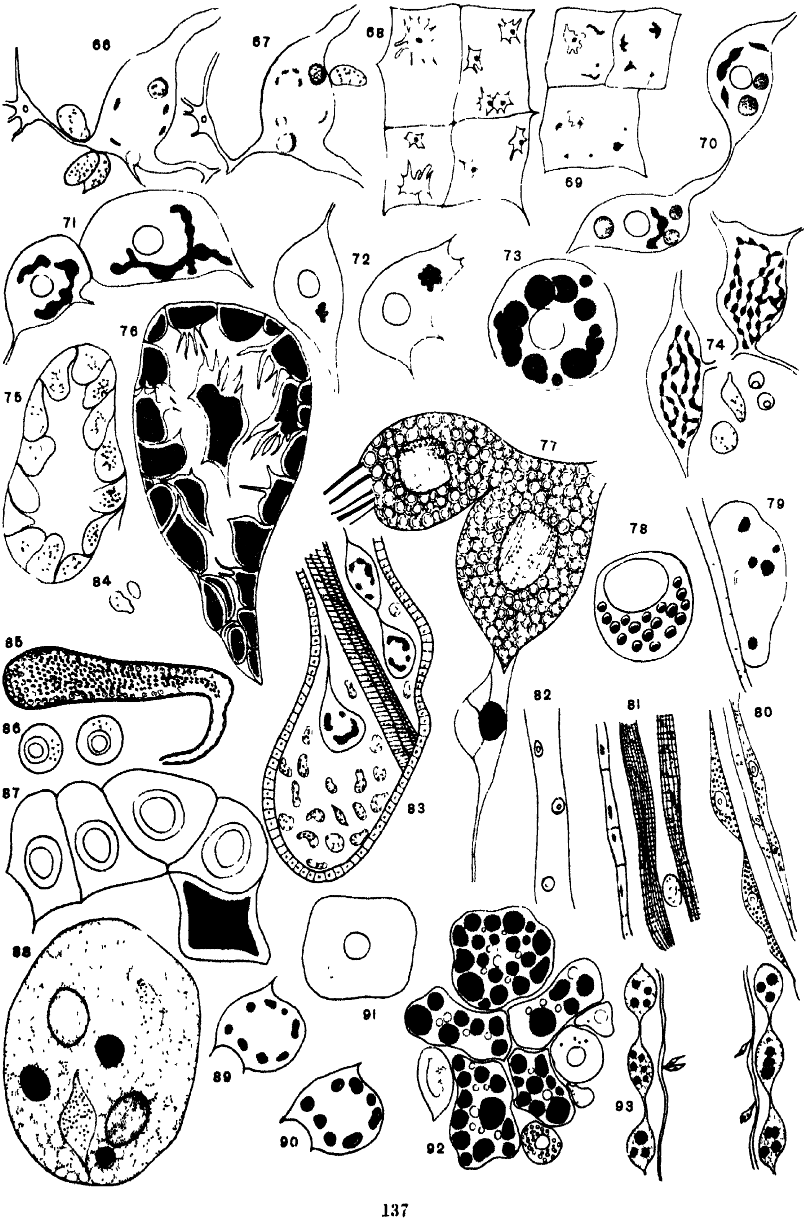
89 Grasshopper blood cell in vitro. Polychrome staining of neutral-red bodies cannot be shown.

90 Same cell as in figure 89, three minutes after exposure to osmic vapor. The color from the neutral-red bodies is fading rapidly, leaving only the osmiophilic rings.

91 Oenocyte in dragon-fly larva. Neutral-red bodies are in alkaline range.

92 Fat-cells and blood cells from Culex larva.

93 Pericardial cells on either side of dorsal blood vessel in Culex larva.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CYTOPLASMIC INCLUSIONS OF TRICHAMOEBA AND THEIR REACTION TO VITAL DYES AND TO OSMIC AND SILVER IMPREGNATION

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ONE TEXT FIGURE AND ONE PLATE (NINE FIGURES)

AUTHOR'S ABSTRACT

In Trichamoeba sp. two types of inclusions are recognized on the basis of reaction to vital dyes and to methods of osmic and silver impregnation. Globular inclusions, which are stained selectively with neutral red, may be blackened under direct observation by exposure to osmic vapor in hanging-drop preparations and demonstrated by osmic and silver impregnation. Rod-like and granular mitochondria, stainable vitally with Janus green, may be distinguished from the neutral-red globules in preparations stained with a mixture of Janus green and neutral red, and are demonstrated by Regaud's chondriosome method.

INTRODUCTION

It has been pointed out by Bowen ('28 c) that little has been learned thus far about the Golgi apparatus of the Sarcodina. Golgi bodies have been described in two parasitic species—Endamoeba gingivalis (Causey, '25) and Endamoeba blattae (Hirschler, '27)—but little work has been done on the cytoplasmic inclusions of free-living amoebae. It has been found that in certain species of Sporozoa (Joyet-Lavergne, '26; Cowdry and Scott, '28) and Mastigophora (Hirschler, '27; Lwoff and Lwoff, '29; Hall, '29 a, b) there are present cytoplasmic inclusions which may be stained vitally with neutral red and demonstrated also by methods of osmic or silver impregnation. The present investigation was undertaken in order to determine whether or not similar inclusions may be demonstrated in a free-living amoeba, and also whether or not such inclusions, if present, resemble in any way the Golgi material previously described for Endamoeba gingivalis and Endamoeba blattae.

MATERIAL AND TECHNIQUE

Material from cultures containing Trichamoeba sp. was concentrated by centrifuging, and subsequent procedures were completed with the amoebae still in the centrifuge tubes. For keeping the tubes in darkness during osmication and other procedures, large glass tumblers were coated with a mixture of paraffin, beeswax, and graphite. Halves of Petri dishes coated with the same preparation were used to cover the tumblers. In osmic impregnation the methods of Mann-Kopsch (Weigl) and Kolatchev have been used, material being osmicated at room temperature in the former method and at 35°C. in the latter. After impregnation, some of the material, in each case, was bleached with hydrogen peroxide and some with turpentine. The Da Fano method of silver impregnation was used also. For detailed accounts of the Golgi methods used, the reader is referred to Bowen's ('28 a, b, c) papers on technique. In addition to methods of impregnation, some material was prepared according to Regaud's method for chondriosomes (using Regaud's formalin-bichromate fixative).

In vital staining, neutral red was made up as a stock solution of 1 per cent in absolute alcohol, while Janus green B was prepared as a saturated solution in the same solvent. In the case of neutral red, a dilution of one part stock solution to fourteen parts absolute alcohol has been found useful for amoeba and also for several flagellates. This is approximately the dilution used by Dawson ('28, '29) in his work on blood cells. In preparations made with this dilution of neutral red the amoebae have, in several instances, shown no loss of activity or other indications of injury after twenty-four hours in sealed preparations. A mixture of Janus green (one part 1:15 dilution) and neutral red (two parts 1:10 dilution) has been used to stain the mitochondria and the neutral-red globules at the same time. Slides were filmed with the dye and then allowed to dry. In order to secure even filming, it was usually necessary to warm the slide over a flame for an instant before adding the dye. A drop of culture material was placed on the filmed slide and a cover-slip sealed in place with melted vaselin.

In the interpretation of observations, Dawson ('29) has pointed out that both the concentration of the dye used and the time of exposure must be considered. The use of too concentrated solutions or prolonged exposure to the dye may induce injury reactions which mask the selective staining of particular inclusions. On the other hand, a too dilute solution of the same dye may also act as a limiting factor in vital staining. In the present case it was assumed that the dye was not too concentrated when no increase in number of vitally stained inclusions was apparent after about the first fifteen minutes and when the amoeba showed no loss of activity or other signs of injury during the period of observation. Such criteria, however, merely prevent the use of too concentrated solutions, and offer no measure of possible limitations in the other direction.

INCLUSIONS STAINED VITALLY WITH NEUTRAL RED

In neutral-red preparations examined, in different instances, from two to ten minutes after the organisms were placed on the slide, the amoebae were found to contain small globules stained with the dye (fig. A, 1, 2). In some specimens the majority of the globules occurred singly, with few aggregates; in other instances, practically all of the neutral-red globules were seen in aggregates (fig. A, 2). In either case the globules were scattered more or less uniformly through the endoplasm. In active amoebae the neutral-red globules were carried along in the endoplasmic currents accompanying formation of pseudopodia.

In a number of preparations in which the amoebae were active after twenty-four hours, there was no change in the appearance of the globules. In a few cases, however, normally active amoebae showed globules from which most of the neutral red had disappeared; a trace was apparent, but the color was very faint as compared with that seen during the first four or five hours in the same preparations.

Since other preparations, made up with the same sample of dye in the same dilution, did not show this partial loss, or 'reduction,' of neutral red, an explanation is not yet at hand.

In addition to the neutral-red globules, the contents of the food vacuoles were often stained more or less faintly. The

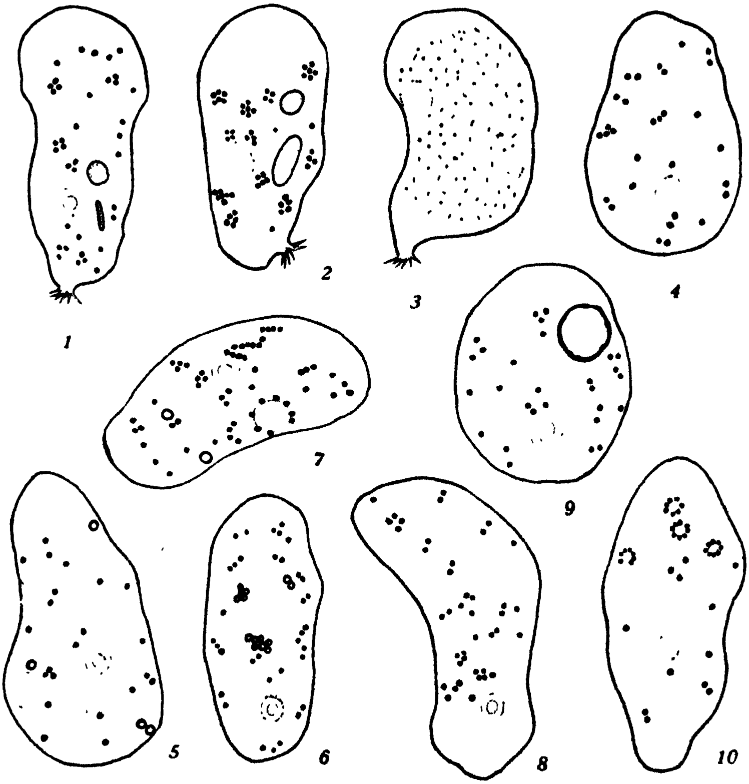

Fig. A Trichamoeba sp., sketches somewhat diagrammatic. Nucleus (and contractile vacuole in 2, 3, and 7) indicated by dotted lines. 1 to 3. Vitally stained amoebae. 1. Neutral-red preparation; outlines of food vacuoles omitted around two food particles. 2. Neutral-red preparation, showing most of neutral-red globules in aggregates; two food particles. 3. Rod-like and granular inclusions stained vitally with Janus green. 4 to 7. Mann-Kopsch material bleached in hydrogen peroxide. 8 to 10. Mann-Kopsch material bleached in turpentine. Several small blackened vacuoles shown in 5 to 8. In 9 a large blackened vacuole, presumably the contractile vacuole, is shown. In 10, note blackened globules adherent to four vacuoles, the walls of which were not impregnated.

contractile vacuole, however, was not affected; nor did numerous small granules and short rod-like inclusions react to neutral red.

REACTION OF NEUTRAL-RED GLOBULES TO OSMIC VAPOR

Cover-slips were filmed with neutral red and a drop of culture material added. After examination (organisms up), to determine staining of the neutral-red globules, the preparations were inverted as hanging-drops over osmic solution in depression slides. Cover-slips were sealed in place with melted vaselin. On examination of such preparations, the cytoplasm of the amoebae showed a light pink tint, due to diffuse penetration of neutral red after death of the organisms. The color of the neutral-red globules, however, had changed to a slightly bluish-red. After thirty to sixty minutes, the change from red to a purplish color was more noticeable. Six hours later, the globules appeared as light gray bodies, contrasting with the pale cytoplasm from which the pink tint had largely disappeared. After about twelve hours, the globules were light to medium gray in color, and in many amoebae the cytoplasm showed no trace of neutral red. These early changes in appearance of the neutral-red globules were perhaps due, first, to an initial change in reaction (apparently a decided drop in pH) which altered the color of the dye absorbed by the globules; and, secondly, to a reduction of neutral red as the osmic vapor began to take effect on the globules.

Subsequent examination of the amoebae at half-day intervals showed a progressive darkening of the globules, and by the end of the fourth to sixth day, the inclusions were definitely blackened in most specimens. Thus the globules in Trichamoeba which are stained selectively with neutral red are also positive in their reaction to osmic, and this reaction takes place slowly just as in the reaction of the metazoan Golgi material to osmic acid (Owens and Bensley, '29).

INCLUSIONS IMPREGNATED WITH OSMIC AND SILVER

In material impregnated after the method of Kolatchev (figs. 1, 2), small globular inclusions are blackened. These are similar in size and distribution to the inclusions which are stained vitally with neutral red. The inclusions remain blackened after bleaching with hydrogen peroxide (fig. 1) as well as with turpentine (fig. 2). Some of the globules are seen in aggregates; others are scattered singly through the cytoplasm. The contractile vacuole and other vacuoles were not blackened in the Kolatchev material examined. In preparations bleached for less than an hour in turpentine, a few small partially bleached inclusions were occasionally seen. It is possible that these inclusions were mitochondria.

In Mann-Kopsch (Weigl) material small globules, similar to those seen in Kolatchev material, are blackened (figs. 3, 4). They are present both in material bleached in hydrogen peroxide and in that treated with turpentine. In the former material (fig. A, 5 to 7) small vacuoles—two, three, or more in number—were blackened in many amoebae. This condition is similar to that reported in several flagellates (Hall, '29 a, b), in which Mann-Kopsch impregnation followed by hydrogen peroxide often results in preparations showing blackened vacuoles. In the preparations of Trichamoeba examined such blackened vacuoles were less common in material treated with turpentine. A very few amoebae showed what appeared to be a blackened contractile vacuole (fig. A, 9), but in the great majority the contractile vacuole, where it could be identified, showed no reaction to osmic (fig. A, 5).

In a few instances an interesting grouping of the osmiophilic globules was observed (fig. A, 10), in which a number of blackened globules were seen adherent to the outer surfaces of vacuoles. Although the walls of the vacuoles were not blackened, the structures were interpreted at first glance as blackened vacuoles; they would probably have appeared as heavily impregnated vacuoles if bleaching had been less effective. Thus it is not certain that all of the 'blackened vacuoles' seen in Mann-Kopsch preparations of amoebae are really the result of osmic impregnation of the vacuole wall itself.

In material impregnated with silver (Da Fano method) small globules are seen scattered through the cytoplasm, singly or in small aggregates (figs. 5, 6). These inclusions are similar in both size and distribution to the globules which are stained vitally with neutral red and which react to osmic impregnation. Most of the inclusions are homogeneously blackened, as in osmic preparations, but some of them (fig. 5) appear as crescents, or almost complete rings, resembling rather closely the Golgi bodies described by Hirschler ('27) in Endamoeba blattae. It is possible that such inclusions in Trichamoeba are the result of incomplete fixation, since they were not noted in material in which the time of fixation had been doubled (fig. 6). Similarly, it has been shown by Parat ('28) that such crescent-shaped inclusions ('dictyosomes') may be produced in gland cells by insufficient fixation in the Da Fano method, whereas longer periods of fixation result in the production of homogeneously blackened globules.

MITOCHONDRIA

In Janus-green preparations (fig. A, 3) a number of small inclusions, presumably the mitochondria, are stained a bluish color within five to ten minutes, and remain stained for thirty minutes or more. In some specimens the mitochondria were chiefly granular, with only a few rod-shaped forms, while in other instances the rod-like mitochondria seemed about as numerous as the granular ones. In the small specimens observed the mitochondria were chiefly granular. In all amoebae observed the mitochondria were distributed more or less uniformly throughout the endoplasm, and they showed brownian movement in nearly all parts of the organism during locomotion.

In preparations stained with a mixture of Janus green and neutral red, two types of inclusions, differing in staining reaction, were seen. The globules stained with neutral red in this mixture appeared identical with the inclusions seen in preparations stained only with neutral red. The neutral-red globules were less numerous than the inclusions stained with Janus green, and most of them were somewhat larger than the mitochondria.

In material prepared according to Regaud's method for demonstration of chondriosomes, rod-like and granular inclusions were seen in the larger amoebae (figs. 7, 8). In smaller forms the inclusions were mostly granular and were relatively few in number, sometimes no more than eight or ten. The appearance and distribution of the inclusions seen in Regaud preparations corresponded to the appearance and distribution of the inclusions stained vitally with Janus green.

DISCUSSION

The Golgi material described by Hirschler ('27) in Endamoeba blattae is somewhat similar in morphology and distribution to that found in certain Sporozoa (Joyet-Lavergne, '25, '26; Hirschler, '27) and several species of Mastigophora (Lwoff and Lwoff, '29; Hall, '29 a, b).[1]

In Endamoeba gingivalis, however, Causey ('25) has described filamentous and net-like inclusions which he considers "typical examples of the Golgi apparatus of metazoan cells." These inclusions are said to be formed by the elongation of crescent-shaped bodies, which in turn are derived from the walls of food vacuoles by 'concentration of material' on one side, 'while the other disappears.' On the basis of the evidence presented in his paper, it would seem that Causey has not entirely obviated the possibility that his filamentous 'closed structures' and net-like inclusions may be bacterial filaments which the amoebae had ingested. Furthermore, in view of the technique used (osmic fixation followed by Regaud's hematoxylin, or Bensley's acid fuchsin-methyl green) it is not absolutely certain that any of the inclusions he has described are true Golgi material, since Bowen ('28 c) has pointed out that, "under ordinary circumstances, these methods would not be expected to demonstrate Golgi material (except in germinal tissues) in metazoan cells. . . .'' At any any rate, Causey's observations on Endamoeba gingivalis might well be checked by the more generally used methods for demonstration of the Golgi apparatus.

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[1] For surveys of the literature the reader is referred to the papers of Hirschler ('27), King ('27), and Bowen ('28 c).

In Trichamoeba sp. it has been found that the inclusions demonstrated by osmic and silver impregnation are discrete globular inclusions, or occasionally crescent-shaped structures (Da Fano) which resemble the Golgi bodies described by Hirschler in Endamoeba blattae. In vital staining, globules similar in size and distribution to those seen in impregnated material are stained selectively by neutral red. These neutral-red globules may be distinguished from the mitochondria by staining the amoebae vitally with a mixture of Janus green and neutral red. Furthermore, these globules may be stained vitally with neutral red and then blackened gradually by exposure to osmic vapor in hanging-drop preparations. Thus it appears probable that the globules which are stained selectively with neutral red are the ones which are blackened in methods of osmic and silver impregnation. Similar cases have been reported for several Sporozoa (Joyet-Lavergne, '26; Cowdry and Scott, '28) and Mastigophora (Lwoff and Lwoff, '29; Hall, '29 a, b). Investigations now in progress in our laboratory have shown that in certain species of ciliates there are similar neutral-red-stainable inclusions which react to osmic and silver impregnation. Thus, with the present observations on amoeba, it is now known that such inclusions are present in at least some species of each of the four major groups of Protozoa.

In this connection, the observations of Dawson ('28, '29), Dawson and Spark ('29), Parat ('28), and Zweibaum and Elkner ('26) are of interest, because of the similarity in reactions of the inclusions they have described to the neutral-red-stainable, osmiophilic inclusions of Protozoa. Dawson ('28, '29) found that the granules of the segregation apparatus of Necturus were stained supravitally with neutral red, and that they were also blackened specifically in osmic and silver impregnation, methods generally recognized as lymphocytes and monocytes the most convincing evidence is obtained that the neutral-red granules may be impregnated with osmic acid. . . .'' (Dawson and Spark, '29, p. 350). Strikingly similar to the osmiophilic inclusions of amoeba are those described by Zweibaum and Elkner ('26) in connective-tissue cells grown in vitro. These authors described the Golgi material ('système vacuolaire') as consisting of discrete scattered globules which were stained vitally with neutral red and impregnated with osmic and silver. And, like the osmiophilic inclusions of amoeba, the Golgi material of these connective-tissue cells ''n'est pas localisé dans la cellule: il peut occuper une position très différente dans les cellules in vitro et probablement aussi in situ, en relation avec la forme et les mouvements de la cellule.''

The inclusions described in Trichamoeba are thus similar to the osmiophilic inclusions of certain Sporozoa, Mastigophora, and Infusoria, and to the Golgi material of certain metazoan cells, in their reactions both to neutral red and to methods of osmic and silver impregnation. The Golgi material of Sporozoa has for some time been recognized as such by metazoan cytologists, and for this reason it seems permissible to conclude that the neutral-red-stainable inclusions of Trichamoeba should be considered Golgi material in the sense of the term as applied to Protozoa. Whether or not such inclusions are homologous with the Golgi apparatus of metazoan cells is a question which can hardly be discussed profitably in view of the present limited knowledge of protozoan Golgi material. Nevertheless, it may be pointed out that these inclusions, at least in Trichamoeba, react to both osmic and silver impregnation, methods generally recognized as standard technique for demonstration of Golgi apparatus in Metazoa, and are also stained selectively in neutral red, thus resembling the metazoan 'vacuome' (Parat, '28; Owens and Bensley, '29).

SUMMARY

In Trichamoeba sp. two types of inclusions are recognized on the basis of reaction to vital dyes and to methods of osmic and silver impregnation. Globular inclusions, which are stained selectively with neutral red, may be blackened under direct observation by exposure to osmic vapor in hanging-drop preparations and demonstrated by osmic and silver impregnation. Rod-like and granular mitochondria, stainable vitally with Janus green, may be distinguished from the neutral-red globules in preparations stained with a mixture of Janus green and neutral red, and are demonstrated by Regaud's chondriosome method.

LITERATURE CITED

BOWEN, R. H. 1928 a The methods for the demonstration of the Golgi apparatus. II. Silver and gold methods. Anat. Rec., vol. 39, pp. 85–136.

——— 1928 b Idem. III. Methods of osmic impregnation. Ibid., vol. 39, pp. 231–284.

——— 1928 c Idem. VI. Protozoa. The vacuome. Plant tissues. Ibid., vol. 40, pp. 225–276.

CAUSEY, D. 1925 Mitochondria and Golgi bodies in Endamoeba gingivalis (Gros) Brumpt. Univ. Calif. Publ. Zool., vol. 28, pp. 1–18.

COWDRY, E. V., AND G. H. SCOTT 1928 Études cytologiques sur le paludisme. III. Mitochondries, granules colorables au rouge neutre et appareil de Golgi. Arch. Inst. Pasteur (Tunis), T. 17, pp. 233–252.

DAWSON, A. B. 1928 The 'segregation apparatus' of the amphibian erythrocyte and its possible relation to the Golgi apparatus. Anat. Rec., vol. 39, pp. 137–153.

——— 1929 A further study of the reaction of the amphibian erythrocyte to vital dyes, osmic acid, and silver salts, with special reference to basophilia and reticulation. Ibid., vol. 42, pp. 281–299.

DAWSON, A. B., AND C. SPARK 1929 The reaction of the segregation apparatus of the thrombocytes and leucocytes of the blood of Necturus to neutral red and to prolonged exposure to osmic acid. Ibid., vol. 41, pp. 335–357.

HALL, R. P. 1929 a Reaction of certain cytoplasmic inclusions to vital dyes and their relation to mitochondria and Golgi apparatus in the flagellate Peranema trichophorum. Jour. Morph. and Physiol., vol. 48.

——— 1929 b Osmiphilic inclusions similar to Golgi apparatus in the flagellates, Chromulina, Chilomonas and Astasia. (Submitted for publication.)

HIRSCHLER, J. 1927 Studien über die sich mit Osmium schwärzenden Plasmakomponenten (Golgi-Apparat, Mitochondrien) einiger Protozoenarten, nebst Bemerkungen über die Morphologie der ersten von ihnen im Thierreiche. Ztschr. f. Zellf. u. mikr. Anat., Bd. 5, S. 704–786.

JOYET-LAVERGNE, P. 1925 Recherches sur le cytoplasme des Sporozoaires. Arch. d'Anat. Micr., T. 22, pp. 1–128.

——— 1926 Sur la coloration vitale au rouge neutre des éléments de Golgi des Gregarines. C. R. Soc. Biol., T. 94, pp. 830–832.

KING, S. D. 1927 The Golgi apparatus of Protozoa. J. Roy. Micr. Soc. vol. 47, pp. 342–355.

LWOFF, M., ET A. LWOFF 1929 L'appareil parabasal et les constituants cytoplasmiques de Leptomonas ctenocephali Fanth. var. Chattoni Lav. et Franch. (Flagelle trypanosomide). C. R. Soc. Biol., T. 100, pp. 557–560.

OWENS, H. B., AND R. R. BENSLEY 1929 On osmic acid as a microchemical reagent, with special reference to the reticular apparatus of Golgi. Am. Jour. Anat., vol. 44, pp. 79–110.

PARAT, M. 1928 Contribution à l'étude morphologique et physiologique du cytoplasme, chondriome, vacuome (appareil de Golgi), enclaves, etc., pH, oxydase, peroxydase, rH de la cellule animale. Arch. d'Anat. Micr., T. 24, pp. 73–357.

ZWEIBAUM, J., ET A. ELKNER 1926 Sur la système vacuolaire dans les éléments cellulaires de tissue conjonctif cultive in vitro. Arch. exp. Zellforsch., Bd. 3, S. 231–241.

PLATE 1

EXPLANATION OF FIGURES

1 and 2 Trichamoeba showing small globular inclusions blackened; Kolatchev osmic impregnation; former bleached in hydrogen peroxide, the latter in turpentine. × 3240.

3 Mann-Kopsch (Weigl) osmic impregnation; bleached in turpentine. × 3240.

4 Same method as in figure 3; bleached in hydrogen peroxide; note several blackened vacuoles. × 3240.

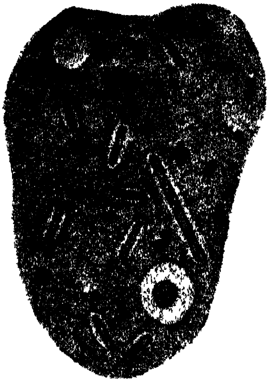
5 Note 'crescents' as well as blackened globules; nucleus not indicated; Da Fano silver method. × 3240.

6 Blackened globules only, same method as in figure 5, but time of fixation twice as long. × 3240.

7 and 8 Inclusions demonstrated by Regaud's chondriosome method. × 3240.

CYTOPLASMIC INCLUSIONS OF TRICHAMOEBA
RICHARD P. HALL

PLATE 1



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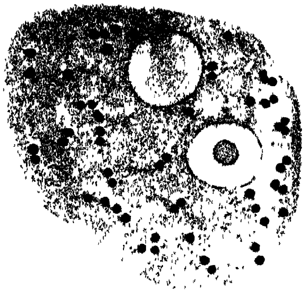

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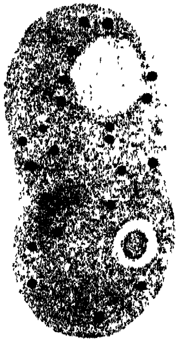

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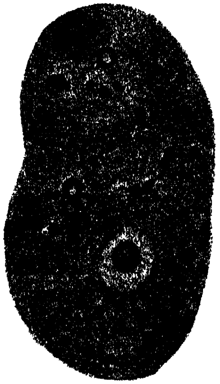

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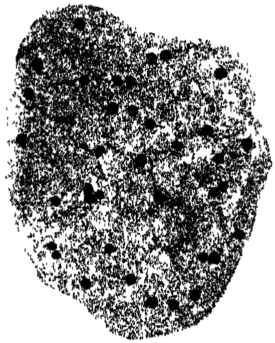

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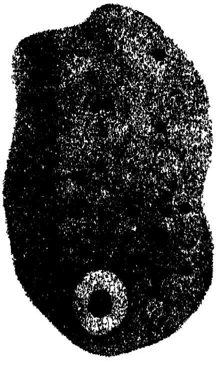

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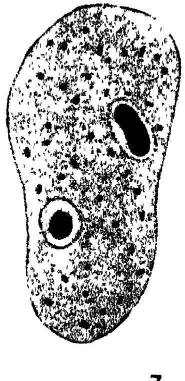

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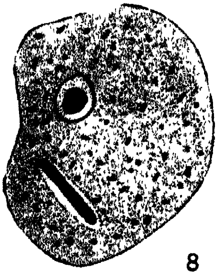

8

THE MORPHOLOGY OF THE PALMAR DIGITAL TRIRADII AND MAIN LINES[1]

INEZ WHIPPLE WILDER

SEVENTEEN TEXT FIGURES AND SEVEN PLATES

AUTHOR'S ABSTRACT

A study is made of the morphological types of the second, third, and fourth interdigital patterns of the human palm and their differential occurrence as a basis for determining the morphological significance of the digital triradii and main lines, which are the final remnants of the pattern boundaries. Certain types of patterns show a large preponderance, with a correspondingly large percentage of a certain morphological value for each digital triradius, although other morphological possibilities than the usual one are discovered in each case. Correlated combinations of rare patterns are presented and their hereditary nature discussed.

INTRODUCTION

All who have worked on the formulation of palmar dermatoglyphics are well aware of the occasional erratic cases in which one is puzzled to locate the triradii of origin of the main lines. Such cases, moreover, frequently involve the presence of interdigital patterns of unusual character which render the tracing of the main lines, even after a decision has been reached as to the identity of their triradii of origin, a problematic matter, while the patterns themselves do not always conform to the established methods of formulation (compare fig. 16 and pls. 2 to 6).

Although the formulation of both main lines and patterns as originally devised by Wilder ('03) and used extensively by him and many other investigators is primarily descriptive rather than morphological in its immediate object, still in these erratic cases the consideration of the morphology of the configuration is a valuable aid to its correct interpretation, and its use should give more than a mere arbitrary basis for formulation. It is the purpose of this paper to furnish such a morphological basis for the formulation of the human palmar configuration in so far as it concerns the relation of the digital triradii and the main lines to the interdigital patterns with which they are associated.

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[1] Contributions from the Department of Zoölogy, Smith College, no. 159.

I wish to express here my indebtedness and that of my late husband, Harris H. Wilder, to Dr. Charles B. Davenport, director of the Carnegie Institution for Experimental Evolution at Cold Spring Harbor, Long Island, and to the management of Howard Orphanage and Industrial School at King's Park, Long Island, for making it possible to collect at the latter institution some years ago the prints of some 145 negro children, inmates of the institution, use of which has been made as supplementary material in this investigation. My thanks are also due to Doctor Davenport and to Dr. Morris Steggerda for the opportunity to look over the prints in the extensive collection made by the latter under the auspices of the Carnegie Institution, of negro, mulatto, and white inhabitants of Jamaica, British West Indies, and for permission to use certain of these as illustrative material.

THE PROBLEM

The single well-marked triradius at the base of each of the four fingers in the usual type of ridge configuration of the human palm is so familiar a feature that we look upon these four triradii as a human characteristic (fig. 1). They may ordinarily be located at a glance and without a thought as to their morphological significance; and the course of the four main lines which arise from them may be readily followed across the palm as expressive of the general direction of ridges of the palm as a whole. Each of these triradii is morphologically, however, the most persistent remnant of the boundary of a primitive interdigital pattern, preserved

Fig. 1 Print of a left hand, showing the usual location and appearance of the four digital triradii and the main lines of the palm which originate from them and are designated, respectively, A, B, C, and D. The numbers are those used in formulating the main-line configuration. From H. H. Wilder, Biol. Bull., vol. 30, p. 137, fig. 1.

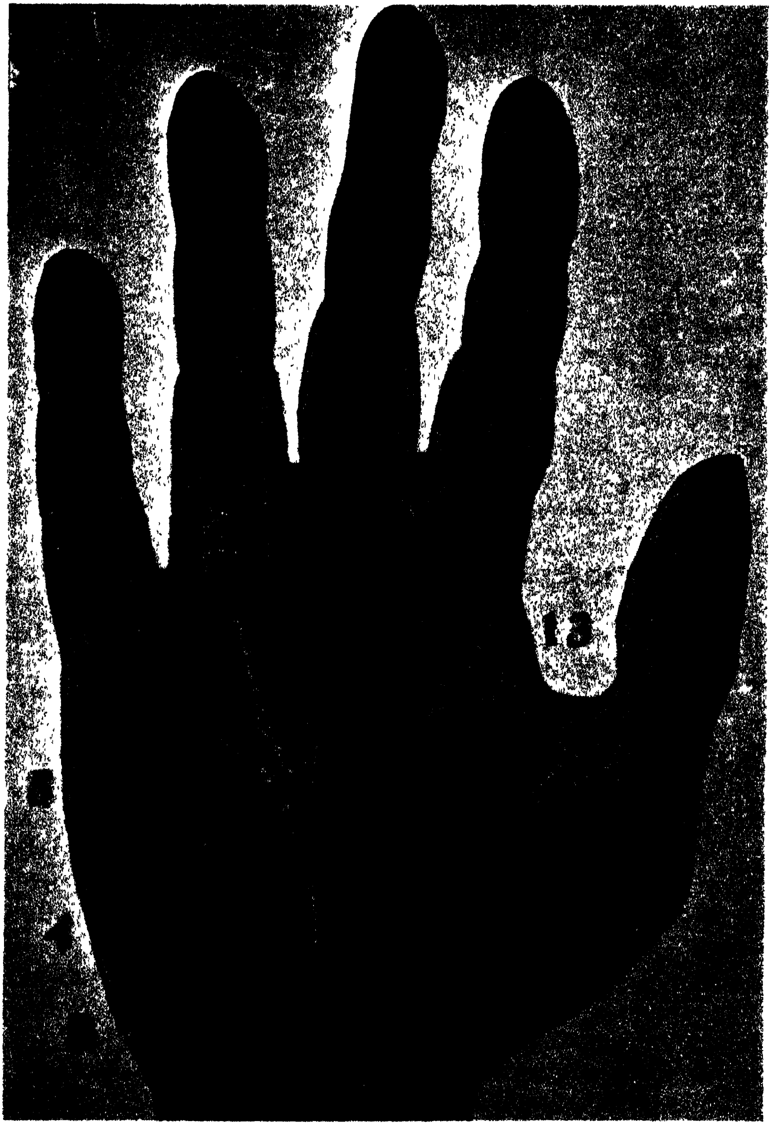

Figure 1

as a distinctive human feature through some combination of factors, apparently mechanically adaptive in character, as the grasping functions of the hand came in the process of evolution wholly to supersede the more primitive walking function. With this change of function the interdigital walking pads which had conditioned the formation of ridges into patterns upon their elevated areas became flattened down and reduced in size almost to the point of extinction. Except, therefore, for the slight disturbances and occasional small patterns upon these reduced interdigital pad areas, the ridges came to take sweeping courses across the extensive palmar surface, following, in general, the direction of the flexion creases which result from the movement of the various separate digits and groups of digits in their opposition not only to the pollex, but to the proximal region of the palm which bears the flattened thenar and hypothenar eminences. Such obvious mechanical factors in the determination of ridge direction are expressed in the use of the term 'friction skin,' which, following the idea first clearly suggested by Hepburn ('95), was proposed by me (Whipple, '04) as more exactly descriptive of the real nature of the ridges than the older term 'papillary' had been.

The persistent and almost universal preservation of the four digital triradii at the bases of the respective digits is unquestionably correlated with the movement of the digits at the metacarpophalangeal articulations. To find evidence for this one has only to note in his own hand the neat manner in which the flexion of each digit gradually folds in the adjacent digital area of the palm along a line parallel with the course of the ridges of this area, until finally the folding reaches the triradius, the two distal radiants of which bound this digital area. The folding developed at the base of each finger as it is flexed is obviously similar in character to that more extensive folding of the skin which in the primitive walking chiridium is necessitated by the presence of the large interdigital walking pads and which forms a complete boundary around them. In these systems of skin folds in connection with the primitive walking pads I found the morphological explanation of the ridge patterns of the Primate chiridium (Whipple, '04). From the general scheme presented in figures 2, 3, 4, and 5, adapted from this earlier paper but with certain modifications of the descriptive nomenclature, it will be seen that, while the first, second, and fourth interdigital patterns have typically three triradii in their boundaries, the third interdigital, located between the more closely associated digits 3 and 4, has a fourth triradius, the distal one, the divergent of which lies between these two digits. Thus the third interdigital pattern is more symmetrically interdigital in position than are any of the others of the series.

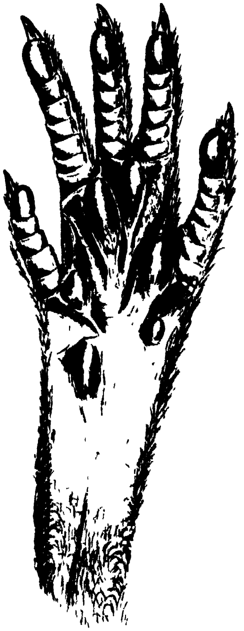

Fig. 2 Left posterior chiridium of Microtus (a field mouse), showing the typical arrangement of walking pads and surrounding skin folds in a primitive mammal. × 5. From I. L. Whipple, Zeits. f. Morph. u. Anthrop., Bd. 7, S. 270, Fig. 1.

It is evident that, with the evolution of the human palmar configuration, profound modifications of this primitive scheme of the interdigital series of patterns and their triradii have

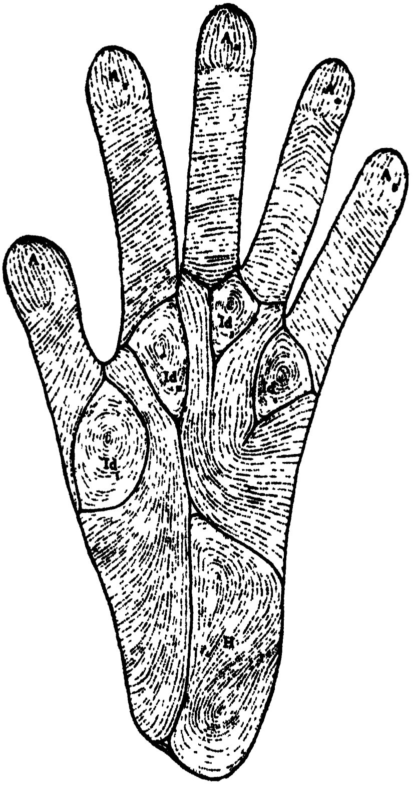

Fig. 3 Ridge configuration of left anterior chiridium of Macacus sp., showing the influence of walking pads upon ridge arrangement. From I. L. Whipple, Zeits. f. Morph. u. Anthrop., Bd. 7, S. 273, Fig. 6.

occurred. The wide separation of the pollex involved in its increased size and perfect opposability has obviously been accompanied by the almost complete obliteration of the first interdigital pattern, although, as has been shown by Wilder ('25), this pattern may occur in a completely primitive condition.

Equally obviously, the digital triradius at the base of the index finger is derived from the second interdigital pattern, those at the bases of the middle and ring fingers from the third interdigital pattern, and that at the base of the little finger from the fourth interdigital pattern. The problem then becomes that of determining which triradius of each pattern it is which persists and whether it is always the same one; in other words, to determine the morphological significance of each digital triradius and of the main line which originates from it.

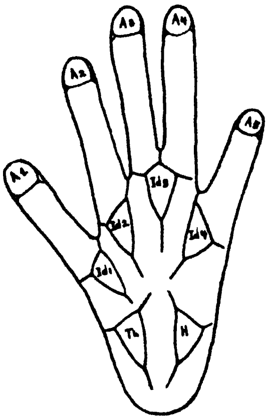

Fig. 4 Diagrammatic scheme for the arrangement of boundaries and triradii of Primate patterns based on the skin-fold arrangement with relation to primitive walking pads (fig. 2). *A 1, 2, 3, 4,* and *5*, apical pads; *Id 1, 2, 3,* and *4*, interdigital pads; *Th.*, thenar pad; *H*, hypothenar pad. Adapted from I. L. Whipple, Zeits. f. Morph. u. Anthrop., Bd. 7, S. 275, Fig. 3.

A preliminary consideration of this question has already been published (Whipple, '04), since incidentally in connection with the discussion of methods of modification of patterns of the Primate palm and sole I described some of the more usual modifications of palmar interdigital patterns and pointed out, by means of several series of examples, the probable morphological significance of the main lines of the human palm and the fact that this is undoubtedly not the same in all cases. I also called attention to the extreme difficulty in establishing the homology, owing to the close similarity of ultimate configurations, when every trace of the pattern except the digital triradius has disappeared.

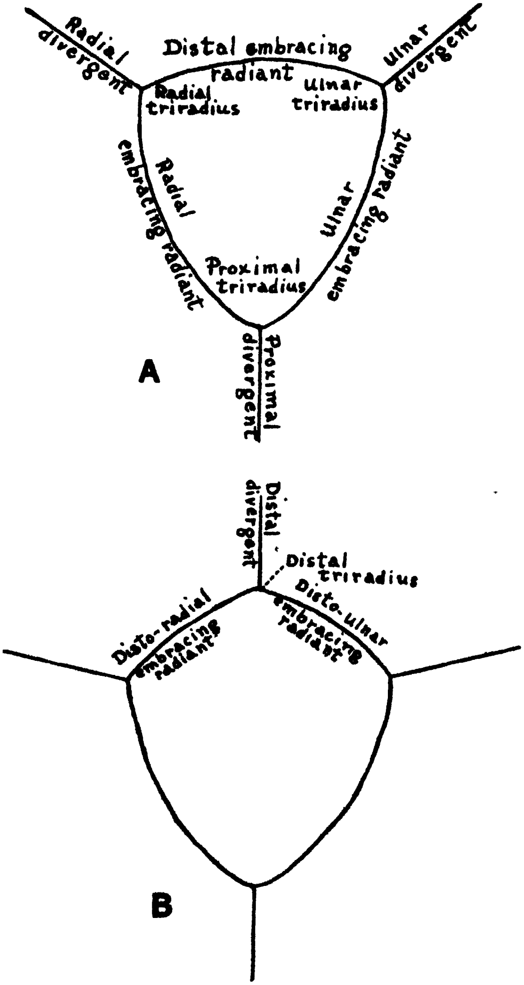

Fig. 5 Diagrams of the boundaries of, A) a typical pattern with three triradii, and, B) a typical pattern with four triradii, with terminology applied as for direct observation of the left hand or for a print of the right hand.

The discussion did not enter into certain possible methods of modification of these patterns, examples of which had not then come under my observation, so that some of the possible homologies of the main lines, if recognized at all, were referred to as purely hypothetical in character, no actual cases having been found to support their claim. With the vastly larger number of prints now available not only in the Wilder Collection, but in others, such cases have now come to notice. These and the conclusions which may be drawn from them constitute the main contribution of the present paper.

It must be emphasized that in the evidence here presented we are not dealing with the actual process of pattern modification, since it is a well-established fact that these patterns are laid down fairly early in the fetus and that whatever influences in the shape of pads or possibly in fetal movements may be brought to bear while the ridges are forming, there is no change even in the minutiae of the configurations once they are established—a process which is completed before birth. Evidence of evolutionary modification, therefore, consists in such seriation of the numberless variations shown by individual configurations as will give complete gradations from a primitive condition to a derived one. Such evidence is convincing only when, as in this case, we are certain which is the primitive end of the series from an evolutionary standpoint.

MATERIAL USED

The material which I have made use of in this paper consists of prints of the hands of members of the Caucasian race (European-Americans) from the H. H. Wilder Collection. For the primary data 200 individuals were used, mainly females, without selection, except to avoid prints which were not clearly decipherable and groups of closely related individuals. Approximately half of the cases taken were prints which had been used previously by Wilder for his early general papers on palm prints. Only a negligible number, however, namely, eleven individuals, had been included in the group of 100 from which data had already been published concerning the occurrence of patterns by Wilder ('22) and made use of by Cummins and Midlo ('26) to combine with their own data concerning the percentile occurrence of the various patterns in the white race.

From these 200 individuals (400 palm prints) were obtained the preliminary data not only as to the percentile occurrence of the three interdigital patterns under consideration, but also the differential occurrence of the various types of each of these patterns, since therein lies the clue to the solution of the question of the homologies of the main lines. Since, however, this purely random collection of 200 individuals failed to include certain rare types of patterns which a general inspection of a much larger number of prints had shown to occur, I went over the prints of some 600 additional individuals, also of the white race, selecting therefrom those which showed more unusual types of patterns, particularly those of so rare an occurrence that they had not appeared at all in the 200 individuals first taken.

Although thus basing my work upon data obtained from the white race, a complete record was also made, for general comparisons, of the interdigital patterns occurring in palm prints of 145 negro children of the Howard Orphanage—material concerning which as yet no data have been published. Moreover, the large Jamaican collection of negroes, mulattoes (browns), and whites (compare p. 154) was looked over for the possible occurrence of types of patterns not already found.

PERCENTILE OCCURRENCE OF INTERDIGITAL PATTERNS

From the palm prints of the random collection of 200 individuals I obtain the following data as to occurrence of the second, third, and fourth interdigital patterns (table 1).[2]

These results do not differ significantly from those already published by Cummins and Midlo ('26), as table 18 on page 497, which presents a summary of the two series of 100 male individuals each, studied by them and the series of 100 females studied by Wilder ('22)—300 individuals in all, or 600 palms. From this table, which includes all the palmar patterns, I have compiled the data referring to the percentile occurrence of the three interdigital patterns under consideration here, and am presenting it in the following form for comparison with my own results (table 2).

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[2] The symbols designating patterns in this and the two succeeding tables follow Wilder ('22).

TABLE 1

DESIGNATION OF PATTERN	LEFT HANDS (200)		RIGHT HANDS (200)		TOTAL (LEFT AND RIGHT) (400)	
	Number	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*
2	4	2.0	11	5.5	15	3.75
3	59	29.5	98	49.0	157	39.25
4'	65	32.5	45	22.5	110	27.5
$4 + 4^t$	71	35.5	59	29.5	130	32.5
Totals	199		213		412	

TABLE 2

Compiled from Cummins and Midlo, '26

DESIGNATION OF PATTERN	LEFT HANDS (300)	RIGHT HANDS (300)	TOTAL (LEFT AND RIGHT) (600)
	Per cent	*Per cent*	*Per cent*
2	2.4	6.6	4.5
3	30.0	55.0	42.5
4'	29.3	18.0	23.6
$4 + 4^t$	35.7	25.3	30.5

TABLE 3

DESIGNATION OF PATTERN	PATTERNS OCCURRING ON LEFT HANDS		PATTERNS OCCURRING ON RIGHT HANDS		TOTAL OCCURRENCE	
	Number	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*
2	4	2.0	11	5.2	15	3.6
3	59	29.6	98	46.0	157	38.1
4'	65	32.7	45	21.1	110	26.7
$4 + 4^t$	71	35.7	59	27.7	130	31.6
Totals	199	100.0	213	100.0	412	100.0

Table 3 gives percentages differing only slightly from those of table 1. Here is shown the relative frequency of each of the three patterns in the total number of patterns occurring: 199 in the left hands, 213 in the right hands, or a total of 412 patterns in the 400 hands examined.

Inspection of these tables reveals two very obvious facts, only one of which, however, has direct bearing upon the problem in hand. This fact is the extremely small incidence of the second interdigital pattern; although, as pointed out by Wilder ('22) it is apparently even more rare in both the Chinese and Japanese. My total of fifteen cases in 400 palms does not give a large amount of material from which to work out the morphology of the associated digital triradius and main line A. Even the additional 1200 or more palm prints which I examined for further examples yielded only seventeen more cases. As a striking contrast to this fact it may be mentioned here that in the 145 negro individuals (290 palms) there were no fewer than forty-nine cases of the second interdigital, mainly as clear-cut types, although a few very definitely identifiable vestiges were included in the count.

The other equally striking fact brought out by these tables is the very conspicuous difference in right and left hands, not so much in the total number of patterns occurring (213 in the right as against 199 in the left) as in the modal occurrence of patterns in the two hands. Thus, in my 200 individuals the third interdigital occurs in 49 per cent of right hands as against only 29.5 per cent of left hands, while for both the false pattern, 4′, and the true fourth interdigital the conditions are reversed, these patterns being considerably more numerous in left hands than in right. Similar conditions are brought out by the summary of the data given by Cummins and Midlo (table 2) with 55 per cent of right hands showing the third interdigital as against 30 per cent of left hands, and with the conditions again reversed for the patterns in the fourth interdigital space. As already stated, these facts do not have so direct a bearing upon the subject of this paper as upon the question of the trends shown in the evolution of palmar dermatoglyphics, which is a subject also under investigation.

TYPES OF INTERDIGITAL PATTERNS

The matter of types of patterns is one which has been carefully elaborated with relation to certain of the primary patterns, notably, of course, the apical patterns of the hand (finger-print types of the criminologist), the hypothenar of the hand, and the first interdigital of the foot (hallucal). Particularly in the case of the apical patterns, the work already published by Mairs ('25) and that which he is still carrying on give us the interrelation of types of patterns showing the different paths by which a series of slight variations involving a difference of only a few ridges may lead from the primitive whorl to the ultimate configuration known as the arch, which is in reality no pattern at all, but an open field. The morphological analysis of types of hypothenar patterns given by Wilder ('26) has been recently further modified and elaborated by Cummins and his collaborators ('29), furnishing a very complete descriptive system, upon a morphological basis, of this extremely variable pattern.

For the interdigital patterns of the hand very little of this analysis of types has been undertaken, and although whorls and loops, with or without triradii in addition to the usual digital ones, have been recognized, the possibility of different morphological varieties of these seems never to have been given serious consideration. Yet it is to this very matter that we must look for our understanding of the morphology of the digital triradii and the main lines.

As a basis for the analysis of the types of these patterns, it will be well to refer to the methods of pattern modification which were attributed by Whipple ('04) to four main causes. They are as follows:

1. Failure of divergents, resulting in triradii becoming either extralimital or obliterated.
2. Reduction of pads, resulting in degeneration of triradii.
3. Flattening of pads, resulting in a deviation from the concentric arrangement of ridges upon the pad area.
4. Fusion of pads, resulting in the coalescence and in the exclusion of triradii.

The first of these causes was treated, in the paper cited, at considerable length as a factor in the modification of interdigital patterns of the palm, not in man alone but in many of the lower Primates, through the encroachment of flexion creases in such a way as to obliterate the proximal divergent of these patterns. These divergents, it will be seen from figures 2, 3, and 4, tend to converge toward the center of the palm. The first interdigital pattern, of rare occurrence in man even as a vestige, has thus apparently suffered, in all cases where it does occur, the obliteration of its proximal divergent and triradius in correlation with the development of the longitudinal flexion crease involved in the opposition of the thumb, the ridges of the region tending to take a course parallel with this crease and thus at right angles with the typical direction of such a divergent. On the other hand, the proximal divergent of the second interdigital falls so nearly in line with either the longitudinal or the somewhat obliquely directed proximal transverse flexion crease that it tends to be preserved in this position rather than obliterated; while the third and fourth patterns, in marked contrast, are subject to modification in the nature of obliteration of the proximal divergent through the influence of the transverse flexion creases, or, since these patterns are in man usually so reduced in size that they lie well distal to even the distal transverse flexion crease, the modification often takes the form of a sharp deflection of the proximal divergent into the general transverse course of the ridges of this distal region of the palm.

It is, however, to the second of the four causes listed above that the more extensive modifications of the interdigital patterns are due. The marked reduction of size of the interdigital pads and of the patterns located upon them, in the line of Primate evolution leading to man, is obvious.

In the previous study which we are reviewing, little emphasis was given to the simple matter of the final complete obliteration of the pattern through the simultaneous approach of the pattern triradii to the center of the patterns (and to each other) as a result of pad reduction, although mention was made of such a possibility in the case of apical patterns. The modifications which were described were those involving an irregular or differential reduction of the pattern whereby the radiants of the triradii connected with it fail to meet and encircle it in a concentric fashion, one or more of the triradii approaching nearer to the center of the pattern than the others. Such triradii embrace fewer ridges than do the radiants of those at the periphery of the pattern, and inevitably this line of variation leads ultimately to the disappearance of the innermost of the triradii, thus converting the primitive whorl into the type of pattern known as a loop, open in the direction of the divergent of the triradius which has degenerated. It follows that the number of possible types of a pattern depends upon the number of triradii typically located in its boundary. Thus the apical patterns with only two associated triradii present a smaller number of possibilities of types from this cause, pad reduction, than do either the interdigital or the proximal series of patterns with their three (and, in the case of the third interdigital, four) triradii.

In whatever direction a looped pattern may open, it follows that by a process similar to that by means of which a whorl was transformed into a loop a second triradius may disappear, and the pattern as a pattern will no longer exist, having been transformed into the configuration known as an arch (which is in reality an open field of gently curving parallel or somewhat converging ridges, the convergence indicating the original location of the two triradii which have disappeared and resulting in a somewhat lenticular or spindle-shaped figure). The remaining triradius will be located on the convex periphery of the curved system of ridges; or, as might happen in the case of the third interdigital, the two remaining triradii may be variously placed in accordance with the identity of the triradii which have degenerated.

As has already been pointed out, the presence of each additional triradius in the boundary of a pattern gives increased possibilities not only for the orientation of the looped pattern formed by the degeneration of the first triradius, but also for the orientation of the arched system of ridges resulting from the degeneration of the second triradius.

As in the case of finger-print patterns, it is possible for many minor variations to occur in the details of the degeneration process. Thus two triradii may degenerate simultaneously, retaining, as they approach the center or core of the pattern, an exact meeting relationship of their radiants, so that the core of the pattern retains its form as a primitive whorl until, with the simultaneous disappearance of the two triradii, it becomes not a loop, but an arch. Or in other cases the embracing radiants of two or more of the triradii may be so interlocked as to form a spiral figure instead of a perfectly concentric whorl, though with such small patterns as these there is little scope for very complicated relationships. Another variant which is frequently seen in the degeneration of these patterns is that in which two triradii retain a location upon the periphery of the pattern, where they may gradually approach each other until they finally fuse or coalesce, thus transforming the configuration into an arch. As in the case of finger-print patterns, it is also possible, in the final stage in the process of the transformation of a loop to an arch, for the configuration known as a tented arch to occur, a single radiant of the degenerating triradius serving as a short axis, about the end of which the system of ridges sharply curves. The formation of such a configuration implies the location of the degenerating triradius somewhat obliquely with the relation to the axis of the loop.

Moreover, even after an arched configuration is formed, it is possible for the remaining triradius (or triradii) to degenerate, thus completely obliterating every trace of the original pattern boundaries, as has obviously occurred in a case like that shown in plate 1, A, where the third interdigital pattern has wholly disappeared, including digital triradii B and C; or in such a case as is shown in plate 1, B, where all traces of the fourth pattern are lacking with the complete suppression of triradius D.

The schemes worked out in figures 6 and 7 show in extremely diagrammatic form some of the simpler possibilities of interdigital-pattern modification along these lines. These will be convenient to refer to later as a basis for interpreting the types of patterns actually found.

The nomenclature which I suggest for such patterns as these is one which is descriptive of the form of the pattern and the method by which it has been modified from the primitive concentric whorl. Thus, in accordance with the usual custom, W is used as the symbol for a whorl and L, as the symbol for loop. The exponent s (W^s) signifies the spiral rather than the concentric form of the whorl, and the exponent v (W^v, L^v) indicates that the pattern is a vestige. The small letters following the W or L are the initials of the respective triradii involved in the pattern (r, radial; u, ulnar; p, proximal; and d, distal), and their order indicates their location with relation to the core or the axis of the pattern, the triradial center which lies nearest the core being the final one. This final letter, therefore, stands for the triradius which, if the method of modification indicated by the form of pattern were continued, would be the next triradius to disappear. When two or more triradial centers are connected by their radiants and are therefore practically, if not exactly, equidistant from the pattern core or axis, the fact is indicated by a hyphen placed between the symbols. The relative order of these hyphenated symbols has no significance, except that, for the sake of uniformity, they are made to read in a clockwise direction. In the case of a four-triradius pattern (namely, the third interdigital), it is possible for two triradii on opposite sides of the pattern core to be practically equidistant from it and still not connected with each other by meeting radiants. Such a relationship I have shown by a dot, instead of a hyphen, between the two symbols. When the open-field stage has been reached and there is no longer any pattern, no initial letter (W or L) is used, but the identity of the remaining triradius or triradii is indicated by their initial letters. The ultimate assumed coalescence or fusion

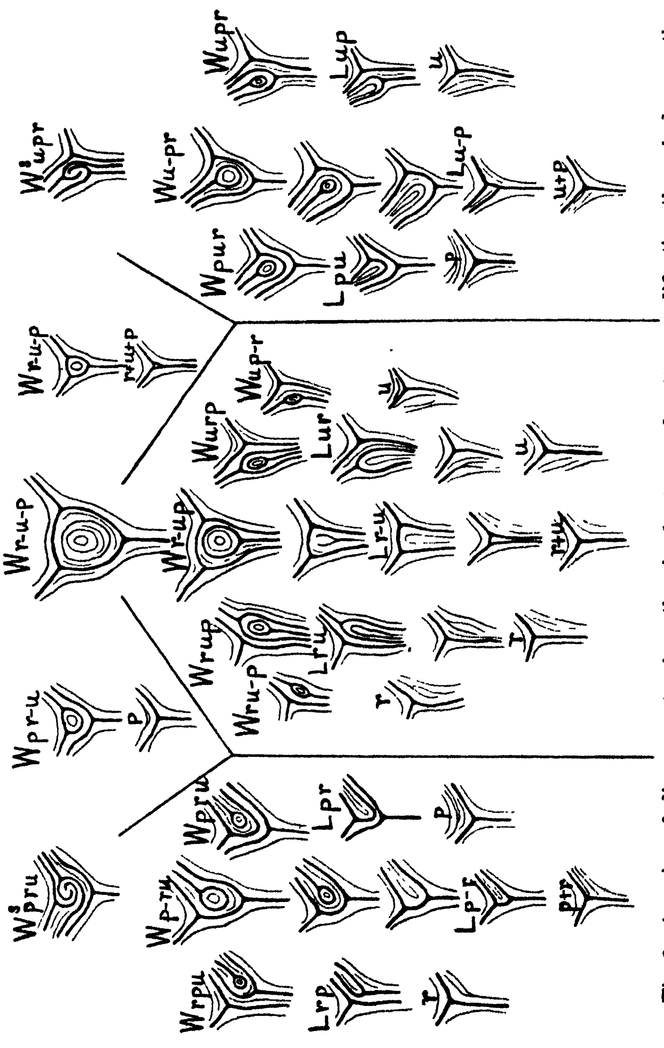

Fig. 6 A series of diagrams, to show the simpler types of pattern modification through degeneration of triradii in the case of a pattern with three triradii in its boundaries (second and fourth interdigital). The nomenclature is explained in the text and is applied as for direct examination of the left hand or for a print of the right hand. The vertical axis of the patterns should be rotated slightly in a contraclockwise direction if used to represent the second interdigital, and in a clockwise direction if used to represent the fourth interdigital.

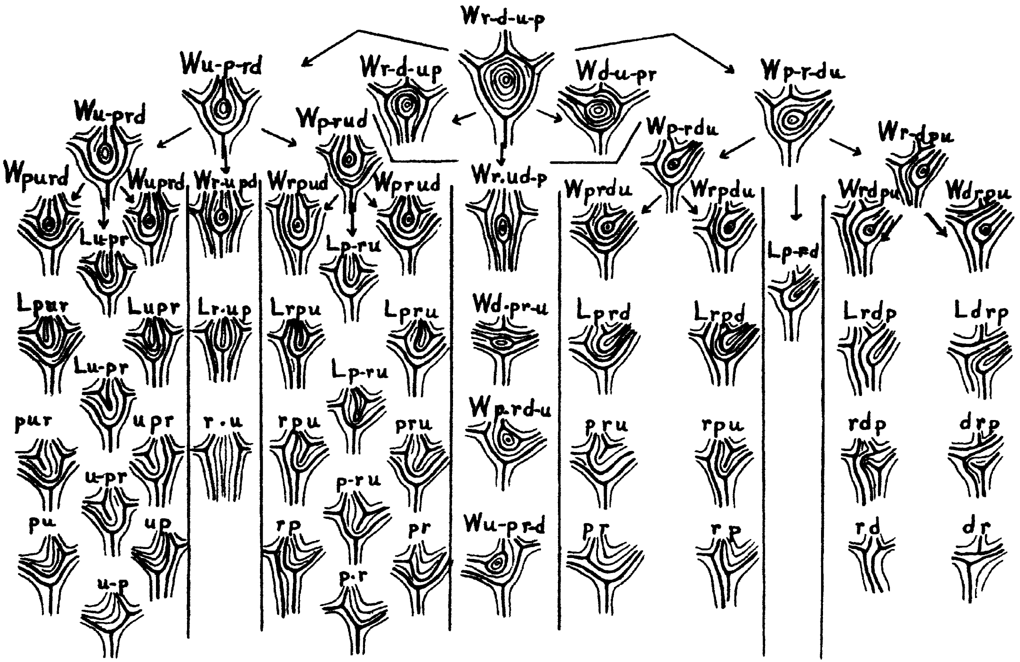

Fig. 7 A series of diagrams, to show some of the simpler types of pattern modification through degeneration of triradii in the case of a pattern with four triradii in its boundaries (third interdigital). The nomenclature is explained in the text and is applied as for a direct examination of the left hand or for a print of the right hand. In its actual application to the third interdigital the vertical axis of the pattern should be rotated slightly in a contraclockwise direction. It will be noted that, for lack of space, only two of the four lines of modification starting from the four types, Wu–p–rd, Wr–d–up, Wd–u–pr, and Wp–r–du, respectively, are carried out to the final stage of open fields. These are the first and last of the four and they comprise the types necessary for reference in the discussion of the cases which were actually found.

of two or more triradii as the culmination of a line of modification is indicated by connecting the symbols for these triradii with plus signs.

In making use of these schematic series, however, it must be remembered that a very potent factor is to be found in the first of the four causes of pattern modification enumerated above, since the obliteration of the proximal divergent reduces enormously the possible number of types of patterns. Also when complete obliteration of the proximal divergent has not occurred, its frequent sharp deflection introduces striking modifications in the total appearance of the pattern.

The two other causes of pattern modification which we are reviewing have less importance in relation to the interdigital patterns than the first two. Nevertheless, the effect of a flattening of the pad area is shown by the fact that, while the region of the degenerating triradii lies near the core of the pattern, there is often a considerable space between this and the outermost boundary of the pattern as identified by the most persistent of the triradii, i.e., the one which will ultimately remain as the digital triradius. Thus are the digital triradii ordinarily held in approximately fixed positions at the bases of the digits, much of the space between them, even when a well-marked pattern is present, being covered by ridges which have the general character of an open field.

Even the fourth cause of pattern modification is seen in certain cases to affect the interdigital patterns, particularly when the two proximal triradii of patterns 3 and 4 are brought closely together or may even coalesce.

With the foregoing general discussion as a basis, we may present the facts concerning the types of patterns which have actually been found in each of the three interdigital areas.

1. *Types of the second interdigital pattern*

Among the small total number of examples of this pattern which were found, only three really distinct types of pattern occur. These and the probable results of the ultimate degeneration of each are shown in figure 8, all drawn as if from prints of right hands. It will be noted that, as already reported by Wilder ('25), no cases of whorls occur. Neither is any case in which the ulnar triradius of the pattern is present, so that not only are all of the patterns loops, but they are loops which open toward the interdigital space.

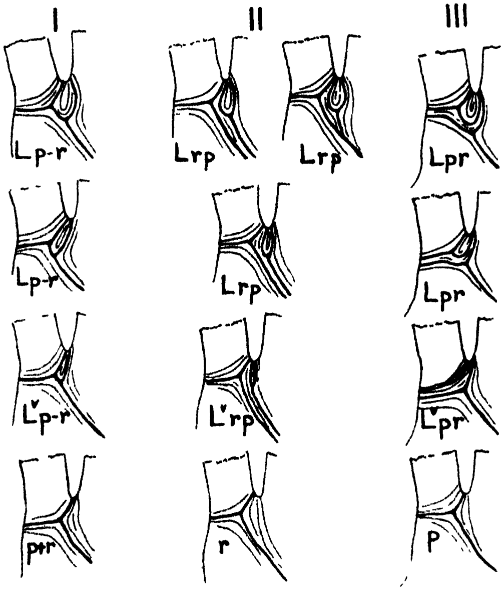

Fig. 8 Diagram illustrating types of the second interdigital pattern which were actually found, arranged in three graded series, or groups, to show the process of degeneration and the final morphological significance of digital triradius and main line A. These are all drawn as from a print of the right hand. The symbols are those used in figure 6 and explained in the text, page 169.

They are, however, types of loops which, because of the relative position of the embracing radiants of the two triradii which are present, lead through the gradations presented by even this small number of cases to an ultimate condition in which the persistent digital triradius has a different morphological significance in each. Thus in group I, in the type designated Lp–r, it will be seen that the diminution of the distance between the radial and proximal triradial centers would lead to their final coalescence into a single digital triradius, the proximal divergent of the pattern becoming the main line A. So long as the pattern is present, however, the digital triradius is the radial one and the main line is a composite of the embracing radiant of this triradius and the radiants of the proximal triradius. Thus, according to the revised methods of formulation of Cummins et al. ('29), main line A would be given a dual formulation, one resulting from following the boundary of the pattern, the other from following the proximal divergent.

In group II, in the types designated Lrp, the proximal triradius is the one approaching degeneration; its divergent, the proximal one of the pattern, if not actually merging, by convergence, with the embracing radiant of the radial triradius, becomes, with the final disappearance of the ridges of the proximal triradius, simply one of the ridges of an open field. The end of this series would leave the radial triradius as the digital triradius, while line A is but the continuation of the radiant which originally embraced the pattern.

Still another possibility is presented by the type of pattern designated Lpr, in group III. Here it is obvious that it is the radial triradius which is on the road to degeneration. Nevertheless, so long as the pattern exists, the digital triradius is the radial one and the main line is that radiant of it which embraces the pattern. The series to which this type of pattern belongs, however, leads to a final condition in which the radial triradius disappears and the only remaining triradius, the proximal one, becomes the digital one, the main line being unquestionably the original proximal divergent of the pattern.

That the ultimate ends of these three series of variations present the same configuration is obvious. Often, however, a definite multiplication of ridges occurs in such a position with relation to the main line as to indicate the type of pattern from which the configuration was derived.

The main line derived from this pattern shows an extraordinary uniformity of course. Line A, not only according to my own data, but also according to the summarized data for 300 individuals given by Cummins and Midlo ('26), practically never has a terminus higher than the ulnar border of the palm (position 5), except in such cases as shown in types Lp–r and Lpr where it encircles the pattern and terminates in position 11, and in all of these cases the proximal triradius is also present and the divergent from this crosses the palm to its terminus on the ulnar margin.

Although it is not in the province of this paper to discuss racial differences, it may be mentioned here that in the negro prints examined, in spite of the larger percentage of occurrence of the second interdigital pattern, there were no types of patterns other than those found in the whites.

Nevertheless, it is not beyond the limits of expectation that other types of this pattern may somewhere come to light. One may well expect to find whorls, and loops of other types than those formed by the degeneration of the ulnar triradius are not beyond the range of possibility.

The following tabulation of the differential occurrence of the types of second interdigital, while based upon far too small a number of cases to rank as conclusive evidence, is at least suggestive of the probable percentage of each of the three homologies of digital triradius and main line A, in those cases in which the pattern itself is absent. In this particular tabulation the total number of patterns found in the Caucasian material is used because the number found in the 200 individuals was so very small and all of those found in the larger number of individuals examined had been recorded, and not merely selected ones.

TABLE 4

Differential occurrence of types of the second interdigital pattern

TYPES	NUMBERS FOUND			PERCENTAGES	HOMOLOGY OF THE ULTIMATE DIGITAL TRIRADIUS AND MAIN LINE A
	Left	Right	Total		
Lp–r	5	6	11	34.4	Radial plus proximal
Lrp	2	13	15	46.9	Radial
Lpr	3	3	6	18.7	Proximal
Totals	10	22	32	100.0	

2. *The fourth interdigital pattern*

In all the prints examined fifteen different types of the fourth interdigital pattern were found—a much larger number than in the case of the second interdigital. Of some of these, moreover, there were two or three well-marked varieties. These types, together with the ultimate configurations which obviously might be derived from them, are shown in figure 9, a and b, all drawn as if from prints of the right hand. It will be seen that they fall into natural groups, the members of which represent to a certain extent stages in the

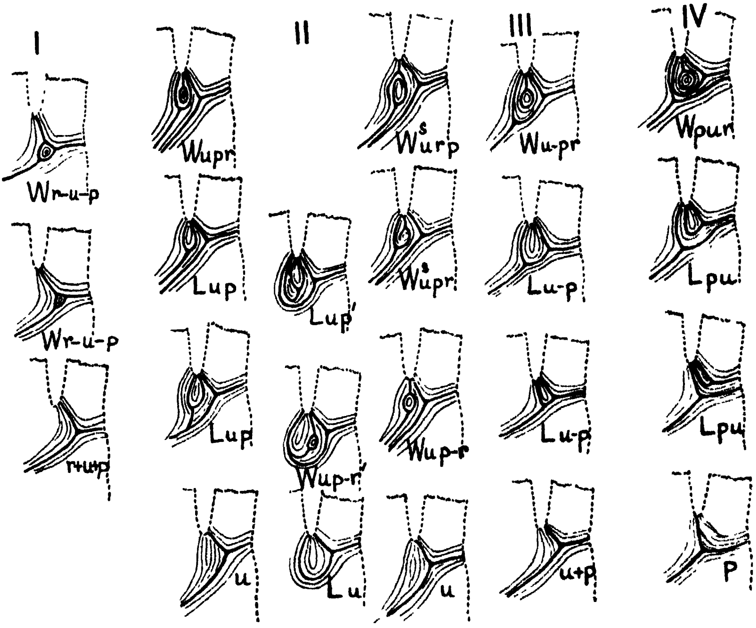

Fig. 9, a and b Diagram illustrating types of the fourth interdigital pattern actually found, arranged in nine graded series, or groups, to show the process of degeneration and the final morphological significance of triradius and main line D. These are drawn as from a print of the right hand. The symbol u^3 in group VI is used to designate the ulnar triradius of the third interdigital pattern which has here become connected with the ulnar of the fourth. Other symbols explained in the text, page 169.

process of transformation of a pattern of the primitive whorl type to an open field.

Group I, comprising typical concentric whorls (or those which are nearly concentric) of small size, shows the possibility of simultaneous approach of all three triradii to a common center with the final complete obliteration of the pattern through their fusion. The result of this fusion is the single digital triradius, while the main line D is the original proximal divergent of the pattern.

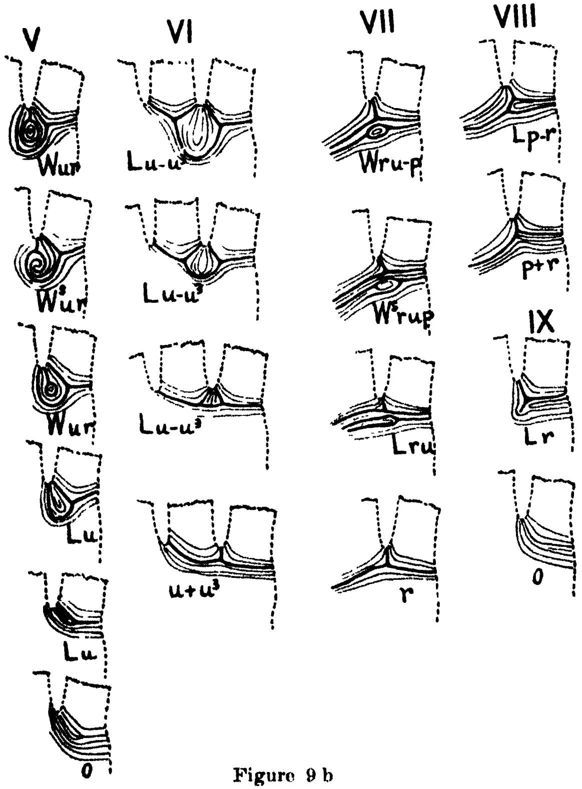

Figure 9 b

Group II comprises a number of varieties of whorls, together with looped patterns such as might be derived from these whorls, all leading finally to an open-field formation, through the degeneration of a second triradius. Among the whorls is to be found the type in which two triradii are simultaneously degenerating—a condition which would lead directly to the formation of an arched configuration or open field without the intermediate loop stage. The remainder, however, show gradations through the stage of the loop which they attain through the degeneration of the radial triradius, the loop in turn becoming an open field through the degeneration of the proximal triradius. In all of this group the most significant fact is that it is the ulnar triradius which remains at the periphery of the pattern, and thus, being located at the base of the digit, constitutes the digital triradius throughout the whole series. The main line therefore, as in group II of the second interdigital, is simply the prolonged embracing radiant of this ulnar triradius. It is interesting to note in this connection that the divergent of the proximal triradius shows a tendency to merge with the main line, while frequently the sharp deflection of the main line itself into a more distal rather than transverse direction contributes to the convergence with the proximal divergent and tends to its obliteration. In other cases (Lup′, Wup–r′) the same forces which cause the main line to take this more sharply curved course seem to have a similar effect upon the proximal divergent which also becomes sharply deflected in a distal direction. Thus, we have a line of pattern modification which, instead of leading to the formation of the usual type of open field consisting of a system of gently curving nearly parallel ridges, finds itself even after the loss of two triradii with the configuration of a secondarily formed looped pattern covering a still larger area than the loops of the true looped pattern of this group. This is undoubtedly correlated with the pressure arising from the transverse flexion of the distal part of the palm and demonstrates one method of suppression of the proximal divergent. The configuration is, however, indistinguishable from such loops as are shown in group V, the further fate of which will be discussed later.

In group III we have a clear-cut line of pattern degeneration with the loss of the radial triradius, followed by the approach of the proximal one to the ulnar until their fusion ends the series, the digital triradius being the result of this fusion and the proximal divergent forming the main line. This is a condition similar to that shown by group 1 of the second interdigital. Until the fusion of the triradii actually occurs, the digital triradius is the ulnar one and the main line is given a dual formulation, one term expressive of the course of the embracing radiants, the other, of the course of the proximal divergent.

Group IV, again starting with a whorl, gives evidence of the formation of a looped pattern through the degeneration of the radial triradius as in the previously described groups. From this point on, however, the course differs, for in this group it is the proximal triradius which remains at the periphery of the pattern, so that, although as long as the ulnar triradius is present, it is undoubtedly to be considered the digital one, and its embracing radiant, the main line, as soon as the last vestige of the ulnar triradius disappears, the proximal triradius automatically becomes the digital one and its divergent the main line.

We come now to group V which again comprises patterns modified by the obliteration of the proximal divergent and triradius. Of the two remaining triradii, the ulnar is in all cases the peripheral one, while by the degeneration of the radial one the pattern becomes converted into the simplest sort of looped pattern which can further degenerate to an open field only through the total suppression of the ulnar triradius. Thus, this series, throughout which the digital triradius is the ulnar one and the main line its embracing radiant, ends with the suppression of main line D and the complete loss of the digital triradius.

It may again be pointed out here that the looped pattern of this series is an identical configuration with that which we have described as secondarily formed under group II through the sharp deflection of the main line and of the proximal divergent which it incloses, or through the obliteration of the proximal divergent by the encroachments of the sharply deflected main line. Moreover, in both cases the simple type of loop formed shows a wide variation in size, so that at one end of the range line D which embraces and bounds the pattern may even meet line C as shown in group VI, Lu–u^3, the whole interdigital area being thus occupied by the fourth interdigital pattern. Such a condition may further lead, through the reduction in size of this inclosed interdigital area, to an actual coalescence and fusion of the two triradii of origin of the main lines D and C, the fused triradius being located interdigitally rather than in either one of the usual digital positions.

It is obvious that we have also here to deal with the question of exactly what constitutes a fourth interdigital pattern. If we consider a configuration bounded by line D meeting line C to be classifiable as a fourth interdigital pattern, we are at once confronted by the problem of the classification of a configuration which may vary from this by only a ridge or two, but in which line D passes to the radial side of line C. This, on the other hand, leaves line C in a recurved position as the boundary of the looped pattern which, whatever its size, conforms to the criterion which we use for the false pattern designated as 4′. Plainly, then, the distinction between such a 4′ and a true fourth interdigital is only an arbitrary one to serve the purposes of a descriptive formulation. There are still other phases of this problem of the morphological significance of pattern 4′, to be discussed later. It may be added here, however, that once the line of variation has carried line D proximal to line C, line D enters the third interdigital area and there may even meet, as in figure 10, the proximal divergent of the third interdigital pattern; or, taking a still more radial course, may meet line B or pass proximal to it.

Groups VII, VIII, and IX comprise the rarest of all the patterns found, the ones, indeed, the finding of which first suggested the need of this investigation. They are all characterized by the fact that the radial triradius retains its peripheral position and is the digital triradius, so that the pattern is located proximal to it rather than in the interdigital space between it and triradius C. In group VII there are whorls, the proximal and ulnar triradii of which are undergoing degeneration practically simultaneously.

In the single type of looped pattern found in this series the ulnar triradius is still present, while the proximal one has degenerated, the small loop thus formed opening proximally, i.e., toward the center of the palm. The transformation of this almost vestigial pattern into an open field is accomplished in such a case by the degeneration of the ulnar triradius, the radial triradius, which throughout the whole series is the digital one, thus being all that is finally left of the pattern, while the continuation of the embracing radiant forms the main line.

Group VIII presents a fragmentary series where obviously the ulnar triradius has degenerated, leaving a small loop in the periphery of which both the radial and proximal triradial centers are located. Such a line of pattern modification would lead to the fusion of these two triradii to form the digital one, while the main line arising from it would be the proximal divergent of the original pattern.

Group IX is also fragmentary, consisting, in fact, of a unique case and that, as it happens, found in a negro print. In this there is a loop which is obviously due to the degeneration of the ulnar triradius. The radial triradius is the digital one, while the proximal triradius has been obliterated through the suppression of the proximal divergent. In this series the next and final step would be the suppression of the remaining triradius, the main line of which here runs only a short course from the triradial center and ends abruptly near the head of the loop. With this suppression the last vestige of the loop would disappear, resulting, as in the series shown in group V, in a suppression of line D, such as is shown in plate 1, B, to which attention has already been called. This print is the left hand of a Bontoc Igorot, and so far as I know is the only case as yet reported of this complete loss of digital triradius and main line D, except those found by Keith ('24) in a number of Korean palms. Naturally, it is impossible to determine by which of the series of variations such an ultimate configuration has been attained.

Inspection of negro prints revealed only the one case, just described, which was a distinctly different type of pattern from those found in the whites. It is interesting to note, however, that among the Jamaican prints of 'browns' there was a case of one of the very rare types under group VII.

It is of interest, now, to inquire into the differential occurrence of the various types of patterns found, as a basis for judging as to what proportion of palms in which the fourth interdigital pattern is lacking, each of the possible homologies of the digital triradius may be applied. Our data for this must in this case be drawn wholly from the types occurring in the random collection of 200, because, unlike the case of the second interdigital, by no means all of the examples of the fourth interdigital occurring in the additional prints inspected were recorded, but only those of unusual character. Since, however, the total number of examples of the fourth pattern occurring in the 200 individuals (400 palms) is 130, we should be able to draw from these fairly reliable conclusions.

The extreme rarity of all those cases comprising groups VII, VIII, and IX, in which the radial triradius is the peripheral and digital one, is brought out by their total absence from the above table. And yet, as already shown, such cases do exist and therefore undoubtedly there are cases in which the digital triradius, in the absence of a pattern, is to be homologized as the radial one, the evidence of this being present in an abrupt distal curve of the main line at its inception, the distal location of the triradial center in an interdigital rather than a typical digital position, and the multiplication of ridges proximal to the line suggesting the vestige of a pattern in that unusual position (compare fig. 16 a and pls. 2 to 5). The occurrence of such patterns seems to be

TABLE 5

Differential occurrence of types of the fourth interdigital pattern

TYPE	NUMBERS FOUND			PERCENTAGES	ULTIMATE HOMOLOGY OF DIGITAL TRIRADIUS
	Left	Right	Both		
Wurp (W^{s}urp)	2	4	6	4.6	Ulnar
Wup–r	0	1	1	0.8	Ulnar
Lup	11	11	22	16.9	Ulnar
Lup′	1	0	1	0.8	Ulnar
Totals	14	16	30	23.1	
Wur	2	1	3	2.3	Ulnar
Lu	32	24	56	43.1	Ulnar (suppression)
Lu–u^{s}	7	10	17	13.1	Ulnar (fusion with ulnar of third pattern to form interdigital triradius)
Totals	41	35	76	58.5	
				Total, 81.6	Ulnar
Wpur	2	1	3	2.3	Proximal
Lpu	10	3	13	10.0	Proximal
Totals	12	4	16	12.3	Proximal
Wu–pr	0	1	1	0.8	Ulnar and proximal
Lu–p	4	3	7	5.4	Ulnar and proximal
Totals	4	4	8	6.2	Ulnar and proximal

correlated with that of certain rare patterns of the third interdigital region, and some further discussion of these will be taken up later.

3. The third interdigital pattern

The types of the third interdigital pattern now claim our attention. Of these there were eighteen distinct types found in my material—a singularly small number when we take into consideration the possibilities in the degeneration of a pattern which has primitively four triradii in its boundaries (compare fig. 7). The reason for the small number is obvious, however, when one notes that the large majority of the types which occur have suffered loss of the proximal triradius through the obliteration of its divergent as a result of the encroachment of systems of ridges parallel with the neighboring flexion creases. The types which were found are shown in natural groups in figure 11, a, b, and c.

In groups I and II have been assembled all the types in which a proximal triradius is present. Group I contains the most primitive example of all, in that it is a whorl with all four triradii present (Wr.upd). Even in this case the crowded condition in this region of the palm shows itself in a convergence and coalescence of the proximal divergent of the pattern with the embracing radiant of the ulnar triradius. This really amounts to an abutment of the latter upon the former so that the two meet each other almost at right angles.

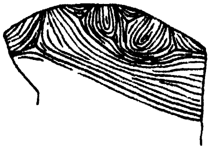

Fig. 10 A tracing of the distal palmar region of the print of the right hand no. 653 of the Wilder Collection. Note that line D passes only slightly proximal to line C, leaving practically the whole of the fourth interdigital area inclosed by the latter and thus technically to be classed as the false pattern 4'. Line D enters the third interdigital space and meets the proximal divergent of the third interdigital pattern.

The remaining types of group I fall into two series of variants, one showing little of the crowding effect of ridges which conform to the distal transverse flexion crease, the other showing in various ways the effect of such crowding. In the first series, group I a, starting with the looped pattern which has resulted from the degeneration of the distal triradius, the pattern undergoes further modification through the degeneration of the proximal one, thus leaving an open field between the two remaining ones, the radial and the ulnar, which are, respectively, digital triradii B and C, while the original embracing radiants of these, as the corresponding main lines, sweep in a nearly parallel course toward the ulnar region of the palm.

Following the other series of variants, group I b, we see the tendency of the proximal divergent to swing more or less sharply in a distal direction, but with varying relationships of ulnar and proximal triradii, so that the four types of loops which were found show in one case (Lrpu) the proximal triradius located peripherally with relation to the ulnar; in the second case (Lru-p) the meeting of the embracing radiants of the proximal and ulnar triradii, the proximal divergent then curving sharply to a terminus on the ulnar side of the

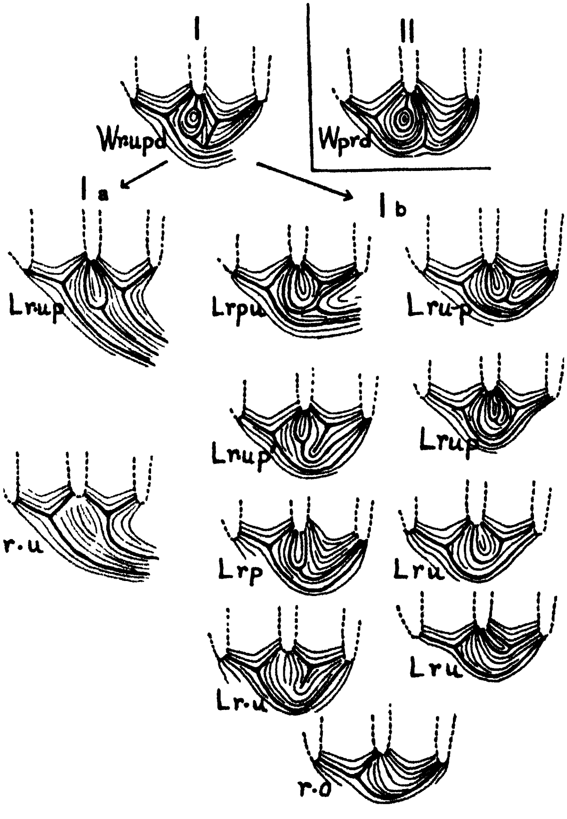

Fig. 11, a Diagrams (see also b and c) illustrating types of the third interdigital pattern actually found, arranged in nine graded series, or groups, to show the process of degeneration and the final morphological significance of digital triradii and main lines B and C. These are all drawn as from prints of right hands. Symbols as explained in the text, page 169.

ulnar triradius; in the third case (Lr.up′) the embracing radiant of the ulnar triradius is cut short, while the proximal divergent bends sharply about its end and passes to the ulnar side of it; and in the fourth case (Lrup) the proximal divergent, bending still more sharply and passing to the radial side of the embracing radiant of the ulnar triradius, outlining in so doing an additional loop, obviously of secondary origin. With regard to the first of these variants, it is one of the cases such as are to be discussed later, in which the boundaries of the third and fourth interdigital patterns, both large,

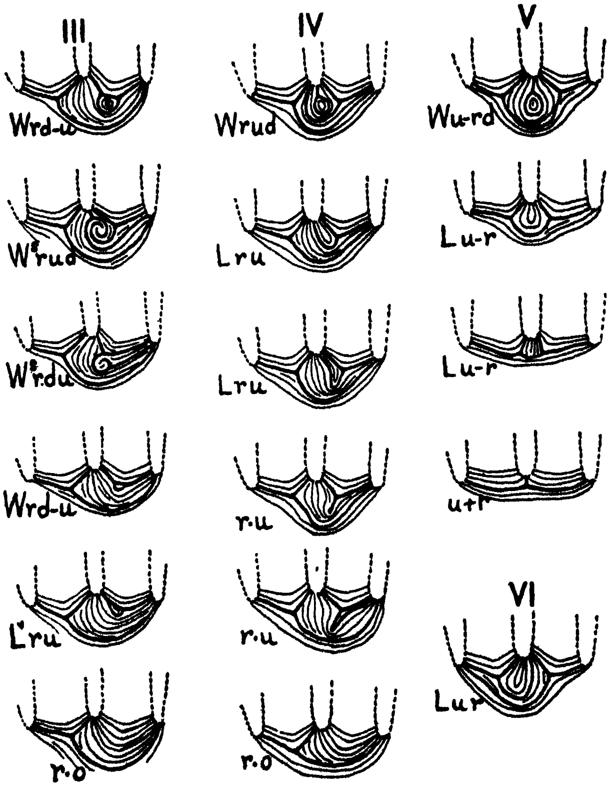

Figure 11 b

tend to coalesce. In the case of the second, although no intermediate types were actually found, it is easy to see that by an approach of the ulnar and proximal triradial centers to the point of fusion not only would the pattern disappear, but also both of the triradii in question. It is one of the possible lines of variation ending in the obliteration of digital triradius C. The third variety, in which the embracing radiant of the ulnar triradius ends abruptly, falls into a series in which with the degeneration of the proximal triradius, the short radiant of the ulnar triradius remains with a system of ridges curving sharply about its end. This configuration, which is that of the typical tented arch, may also be derived through a somewhat different series of variations and is therefore to be found as one of the variants of group IV. Further variation along the same line would eventually lead to a complete suppression of this abbreviated radiant, and with it of the ulnar triradius, which is digital triradius C. The same result is reached if this suppression of the line precedes the degeneration of the proximal triradius, as is apparently the case in the type designated Lrp.

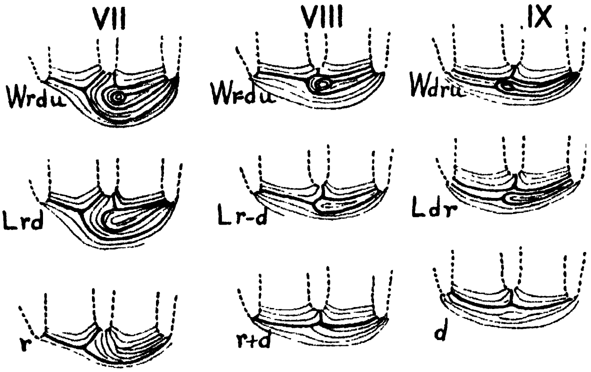

Figure 11 c

In the fourth variety, in which the proximal divergent is much crowded in its sharply curved course, the degeneration of the proximal triradius would leave a looped pattern bounded by the embracing radiant of the ulnar, and, outside of this, of the radial triradius. Such a configuration—a very common one—fits also into another and more usual series of variants, that of group IV, and is, of course, like the tented-arch configuration, indeterminate in its origin once it is established.

The single example given in group II is a whorl of unique type in two respects: the position of the proximal triradius peripheral even to the embracing radiant of the radial one, and the complete obliteration of the ulnar triradius. Thus, digital triradius and main line C are lacking, unless the proximal triradius, in spite of its location, be allowed to play the rôle of digital triradius, its radial embracing radiant becoming the main line. On the other hand, if the order of degeneration indicated by the relative location of the three triradii should be carried out, the series would lead finally to the obliteration of the radial triradius, and the proximal one would then become digital triradius B, its divergent constituting main line B. However, no series of gradations leading to either of these conditions was found.

The types designated as group III are peculiar in that the patterns, which are whorls, are small and have the two triradial centers of the distal and ulnar triradii either equidistant from the core of the pattern or nearly so. In the two examples of spiral whorls it will be noted that careful tracing of the ridges would show that in one case it is the distal triradius of the pattern which is by location the digital one, while in the other case it is the ulnar triradius. All of these patterns, however, grade through a series which, through the diminution in the size of the pattern, results finally in the obliteration of both of these triradii, practically simultaneously, again resulting in the suppression of digital triradius C and its main line.

Group IV is made up of very common types of the third interdigital. The whorl with but three triradii, in the absence of the proximal one, grades into the type of loop which opens distally, in the boundary of which the radial triradius has the peripheral location. The respective homologies of digital triradii B and C are obvious, main line C being the embracing radiant of the ulnar triradius and curving radially and distally to outline a looped system of larger or smaller size, while line B is the embracing radiant of the radial triradius and passes ulnarward to form, in the early part of its course, the outer boundary of the looped pattern. The variations in the size of the loop outlined by line C grade finally into a condition identical with that shown in group I b, in which the line no longer forms a loop, but describes an abbreviated straight course and constitutes the core of a tented arch. It is obvious that variation to the extent of but a single ridge would lead from this configuration to one in which line C turns sharply to take an ulnar and distal course and outlines a loop designated in the accepted formulation as 4′, since it is obviously of secondary origin and is located in the fourth interdigital space. Further variation in this direction might lead to the formation of larger and larger patterns of this secondary type, until the larger part of the fourth interdigital area becomes thus involved. In fact, as pointed out under the discussion of the fourth interdigital pattern, the secondary pattern so grades into the true looped pattern of large size in this area that the final distinction between them must be an arbitrary one.

The abbreviated condition of line C, however, may also, as already pointed out, lead by a direct series of gradations to the complete suppression of the line and its triradius of origin.

Group V includes a series of types which are of interest because they lead through a complete obliteration of the pattern to a coalescence of the radial and ulnar triradii into a single triradius in an interdigital position, with no triradii so located as to conform to the definition of either digital triradius B or C. Both of the main lines corresponding to these are naturally also lacking, although up to the stage of actual coalescence two digital triradii are present and their main lines, though of very abbreviated course, meet each other.

The single type which constitutes group VI represents the only variant of the pattern found, in which the ulnar triradius takes the more peripheral position, thus reversing the usual relationship of the ulnar and radial triradii in the boundaries of the simple looped pattern. Gradations of this type leading to the suppression of the radial triradius were not found. They are, however, by no means beyond the range of possibility.

Groups VII, VIII, and IX include rare types which have great morphological interest in that in all of them the core of the pattern is not located within the area bounded by main lines B and C which is by our ordinary usage designated the third interdigital area. These types are therefore comparable to the fourth interdigital patterns of groups VI, VII, and VIII, the cores of which are proximal to main line D, instead of between it and line C. Moreover, these rare types of both the third and the fourth interdigital patterns occur together in a sufficient number of cases to suggest strongly a correlation between them.

In group VII, of the third interdigital, it is the ulnar triradius which lies nearest the core and is degenerating. Thus, *the distal one, although located interdigitally, becomes by definition digital triradius C, being located nearest to the base of the ring finger. Main line C is therefore formed by its* radial embracing radiant. With the loss of the ulnar triradius, the looped pattern which results takes on the extraordinary relationship of opening in the direction of the fourth interdigital space, and would thus in our pattern formulation be classed as the secondary pattern designated as 4′, although the marked sweep of line C radialward before it curves ulnarward to encircle the looped set of ridges brings the core of the pattern in line with the third interdigital space, while by definition it is not located within it!

The single type given under group VIII differs from the whorl type of group VII only in the location of both radial and distal triradial centers at the periphery of the pattern. As the ultimate result of the degeneration of the patterns of these two series, group VII would lead to the suppression of the distal triradius, hence the loss of digital triradius C and its main line, while group VIII might result in the coalescence of the radial and distal triradii into an interdigital one with the obliteration of both main lines B and C—a condition like the final one in group V, but with a different homology.

In group IX we have the distal triradius persisting at the periphery of the pattern, while the ulnar is the first to degenerate and the radial next. Here again the looped pattern opens toward the fourth interdigital space, although the core of the pattern is in direct line with the middle of the third interdigital space. The embarrassment in formulating, however, comes from the fact that the embracing radiants of the distal triradius cut off both the radial and the ulnar triradii from their typical relationships with the bases of their respective digits upon which their identity as digital triradii is based. Thus, from the beginning of this line of variation these two digital triradii give way to a single interdigital one which persists after the pattern has disappeared.

Throughout all of these series of types one sees that the radial triradius shows an astonishing degree of persistence in its position as a typical digital triradius at the base of the middle finger. Only in those cases where it coalesces with another triradius and becomes interdigital (groups V and VIII) and in group IX, where it degenerates, are there actual exceptions to this. Moreover, aside from these exceptions, there is apparent no tendency to the suppression of the main line B which originates from it, and the only other type in which the conditions are such that there would be a possibility of such suppression are the single types included, respectively, in groups II and VI.

With the ulnar triradius the case is quite different. While usually the lines of variation are such that the ulnar triradius, like the radial one, persists at the base of the digit as the digital triradius, there are cases in which it is supplanted by the distal one (groups VII, VIII, and IX) or in which it coalesces with the radial one (group V) to form an interdigital triradius. More significant than these exceptions, however, is the fact of the obvious tendency through so many of the series, to its final complete suppression, digital triradius and main line C being thus wholly wanting. Only in the series of variants of group I a in which the proximal triradius is present with its divergent lying in a parallel course between lines B and C, both of which sweep ulnarward, is this tendency to obliteration of line C not in evidence. The frequent combination of circumstances of the absence of the proximal triradius and the peripheral location of the radial one with reference to the ulnar makes possible this course of variation leading to suppression. The frequency with which the pattern thus encircled by the embracing radiant of the ulnar triradius is of small size and in one way or another is crowded against the ulnar triradius, leaving often a considerable width of open field between the pattern and the radial triradius, is a further indication of this tendency to suppression of the ulnar triradius.

The following table shows the differential occurrence of the various types of patterns which were found in the 200 individuals (400 palms) of the random collection studied. As in similar tables for the second and fourth patterns, these percentages form a basis for conclusions as to the probable homologies of the digital triradii B and C in those cases in which no patterns are present.

Even in the absence of most of the rare types of patterns from the third interdigitals which occur in this random collection of 400 hands, nearly every possibility of homology of the two triradii in question is represented. Only the loss of both ulnar and radial ones with the persistence of the distal one as an interdigital is lacking from the examples found here.

The most striking fact shown by the tabulation is, however, the enormous preponderance of the very simple type of loop (Lru) in both hands, and the difference in its percentile occurrence in the two hands, its incidence in the right being nearly twice that in the left. This type of pattern, which constitutes 85.5 per cent of all the third interdigital patterns found, is comparable to the predominant type of fourth interdigital, the simple loop, Lu, which constitutes over 43 per cent of all the fourth patterns. Both of these types may, as we have seen, be derived through two different series of variations, and once this stage in the modification of the pattern is attained, any encroachment of the systems of ridges parallel with the distal transverse flexion crease has only the effect of reducing the size of the pattern, while the type remains the same until the tented-arch stage or that of complete obliteration of the pattern, including even the ulnar triradius itself, is reached.

TABLE 6

Differential occurrence of types of the third interdigital pattern

TYPE	NUMBERS FOUND			PERCENTAGES	ULTIMATE HOMOLOGY OF DIGITAL TRIRADII	
	Left	Right	Both		B	C
Lr.up	0	1	1	.6	Radial	Ulnar
Lr.up′	1	0	1	.6	Radial	Ulnar (sup.)
Lrpu	1	0	1	.6	Radial	Ulnar
Wrd-u	4	0	4	2.5	Radial	Distal + ulnar (sup.)
W^{s}rdu	0	2	2	1.3	Radial	Distal (sup.)
W^{s}rud	1	0	1	.6	Radial	Ulnar (sup.)
Wrud	1	1	2	1.3	Radial	Ulnar
Lru	50	85	135	85.5	Radial	Ulnar (sup.)
Wu-rd	0	1	1	.6	Radial	Ulnar
Lu-r	1	4	5	3.2	(Interdigital by coalescence of radial and ulnar)	
Lur	0	4	4	2.5	Radial	Ulnar
Lrd	1	0	1	.6	Radial	Distal (sup.)
Totals	60[1]	98	158	99.9		

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[1] The discrepancy between this number and the corresponding one in table 1 is due to the fact that the example of Lrd included here was classed by definition as 4′ in table 1. Doubtless, many others of this heterogeneous group could be thus transferred to the category of the third pattern, did we but know the history of their formation.

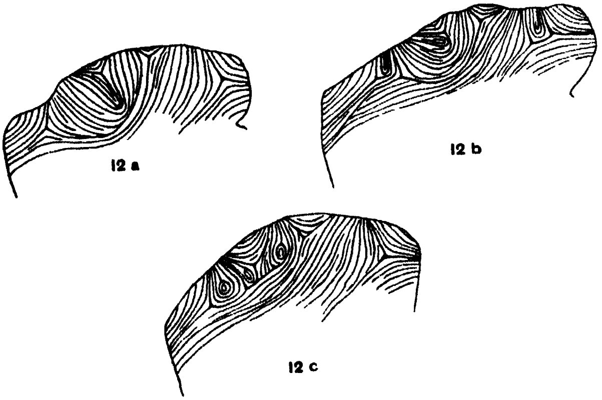
4. *The false pattern (4′) of the fourth interdigital area*

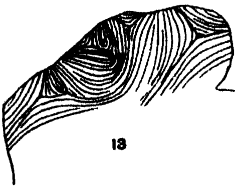
In the course of the above description of types of patterns, we have seen at least four possible methods by which patterns which we have been accustomed to designate 4′ may be derived. These we may now summarize.

The first of these methods lies along the range of variation of the fourth interdigital pattern of the simple loop type which may become so extensive (Lu–u^3) as to include the whole of the fourth interdigital space, so that main lines C and D meet to form its proximal boundary. Continuing this line of variation, there would come the condition in which line C forms the inner boundary of the pattern, line D passing on its proximal side as in figure 10. By definition, this pattern, varying from the true fourth interdigital by possibly only a single ridge, becomes the false pattern 4′—obviously, a purely arbitrary distinction. And yet there are all gradations in the size of such patterns from this which occupies practically the whole of the fourth interdigital space to a loop of a single ridge in width. It is obvious that a 4′ pattern of such a derivation could not exist simultaneously with a true fourth interdigital.

A second method of derivation of 4′ is seen in following the gradation from the stage in which line C is the core of a tented arch to the turning of the line ulnarward, thus embracing a loop, however narrow, in the fourth interdigital space (fig. 12 a). As in the case of the first method of derivation, so here we have the possibility of a complete seriation as to size of 4′, in this case, however, leading toward the inclusion of a larger and larger area of looped ridges. Unlike the first, however, this variety undoubtedly sometimes exists simultaneously with a true fourth interdigital (compare fig. 12 b), and it has been found also coexistent in one case at least with both a third and a fourth (fig. 12 c).

A third method of derivation of pattern 4′ is that incident to the imprisoning of the proximal divergent of the third pattern in such a way that it is bent sharply distalward on the ulnar side of the ulnar triradius of the pattern. This variety, like the second, might be present in combination with the true fourth interdigital pattern and has been found to exist together with both third and fourth (fig. 13).





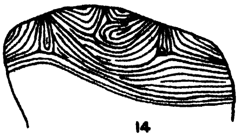


Fig. 12 Tracings of the distal palmar region of three prints of left hands from the Wilder Collection, to show examples of the false pattern 4′ formed by deflection of line C into the fourth interdigital space. a, no. 877, shows the occurrence of 4′, with no other pattern present; b, no. 899, shows the occurrence of 4′, together with a true fourth interdigital pattern as well as a second; c, no. 306, shows the occurrence of 4′, together with both a third and a fourth interdigital pattern.

Fig. 13 Tracing of the distal palmar region of a print of the left hand of no. 613 of the Wilder Collection, to show an example of 4′, formed by the deflection into the fourth interdigital space of the proximal divergent of the third pattern. A true fourth pattern is also present.

Fig. 14 Tracing of the distal palmar region of the print of the right hand of no. 6696 of the Carnegie Jamaican Collection of 'Browns,' to show an example of an obvious third interdigital pattern which has degenerated in such a way as to conform technically to the criterion of the false pattern 4′, in that it is inclosed by line C which loops about, into the fourth interdigital area. A true fourth interdigital is also present.

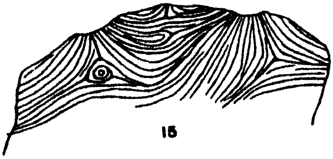
The fourth method of derivation belongs to quite a different category in that here the third interdigital itself undergoes modification of such a type (compare fig. 11 c, groups VII, VIII, and IX) that when the stage of the looped pattern is reached the loop opens out into the fourth rather than the third interdigital space, although the core of the loop is in line with the middle of the third space. Such a loop, unquestionably in reality a true third interdigital pattern, would nevertheless by our definition be classed as the false pattern 4′, since there is no absolute criterion by which one can establish that the digital triradius C is in this case not the usual ulnar one, but the distal one of the third interdigital pattern. This variety might occur together with a true fourth pattern (fig. 14), but never with a third, since it is in reality the third pattern itself.

These four possible lines of variation leading to the configuration 4′ will account for the frequency of occurrence of this pattern, namely, in 32.5 per cent of left hands and in 22.5 per cent of right in the random collection of 200 individuals. Of these, however, only one-tenth occurred together with the fourth interdigital and none with the third, so that, so far as these statistics go, nine-tenths of the cases occurring might have been derived as a variant of the fourth interdigital, and any of them might have been derived from the third. There is no criterion, however, upon which to make such a distinction except in occasional cases which lie near the border line.

5. *Correlation of types of third and fourth patterns*

In the foregoing account reference has been made (p. 194) to the fact of the simultaneous occurrence of the rare or erratic types of the third and fourth interdigital patterns—those, in other words, which are otherwise located than in the third and fourth interdigital spaces, respectively—as these are ordinarily defined, the third between digital triradii B and C and the fourth between digital triradii C and D. Such a case is shown in figure 15.

In addition to such combinations as these, we have examples of the combination of one of the rare types of the fourth pattern with the extremely reduced or wholly suppressed condition of the other as shown in figures 16 a, b, c, and d.



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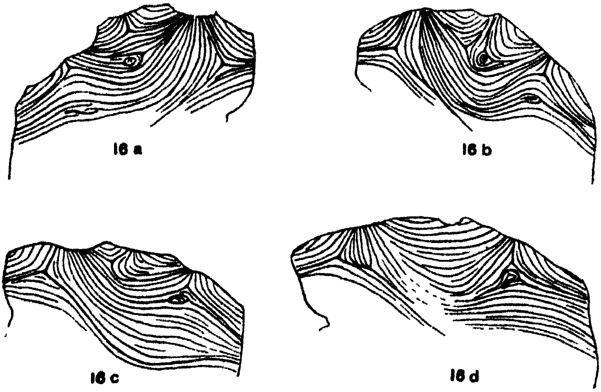


16 a

16 b

16 c

16 d

Fig. 15 Tracing of the distal palmar region of a print of the left hand of no. 1247 of the Wilder Collection, to illustrate the combination of 'rare types' of third and fourth interdigital patterns. Note that the interdigital triradius between the ring and middle fingers is morphologically the distal one of the third interdigital pattern and that neither digital triradius B nor C is present.

Fig. 16 Tracings of the distal palmar region of four prints from the Wilder Collection, to illustrate in the case of the third and fourth interdigital patterns combinations of 'rare types' of one with nearly or quite suppressed conditions of the other. a, no. 401, left; b, no. 401, right; c, no. 1295, right; d, no. 1247, right.

Such combinations as these of patterns that are themselves so unusual would seem to be more than a mere coincidence. The most significant example of their occurrence, however, is seen in the case of a family of five, consisting of the two parents and three sons and shown in plates 2 to 6.

In the case of the three sons in this family two right hands (compare pls. 2, B, and 3, B) possess well-defined fourth interdigital patterns of the type Wru–p, in which the digital triradius D is the radial one of the pattern, while the remaining four hands have in varying degrees the interdigital rather than the typical digital position of triradius D and a multiplication of ridges, particularly well marked in the two hands of one individual, representing a vestigial pattern in the location corresponding to the actual patterns of the first two cases.

As for the third interdigital, one son shows (pl. 2) the rare type Wrdu on both hands, the right in combination with the rare fourth pattern. A second shows in both hands (pl. 3) the complete suppression of the third pattern and the absence of digital triradius and main line C. In the right hand this is in combination with the rare fourth pattern. The third shows (pl. 4) this combination of the obliterated third pattern and the interdigital location of digital triradius D in the left hand, but in the right a fairly typical pattern of the 4′ variety, which, of course, may have been derived from a rare third interdigital or from a suppressed line C deflected toward the ulnar side. To summarize, out of six palms we have five with a combination of either an erratic third and fourth or of an erratic third with a vestige of an erratic fourth, or a totally suppressed third pattern combined with either an erratic fourth or with a vestige of it.

When we examine the father of these three, we find (pl. 5) his right hand with the same type of configuration as the left of two of the sons, while the left hand shows the suppression of the third pattern (with a vaguely represented vestige only of line C), but with a small fourth interdigital of the more usual type Lu–p. If we are looking for further hereditary influence, we find that it is furnished by the mother, in both of whose hands there is the same suppressed condition of the third interdigital with absence of line C. Her digital triradii D, however, show no indication in their location nor, in the course of main line D which proceeds from them, of being morphologically other than the usual ulnar triradius of the fourth pattern. In the absence of these patterns themselves, somewhat vague vestiges are shown in the usual position on the radial side of line D.

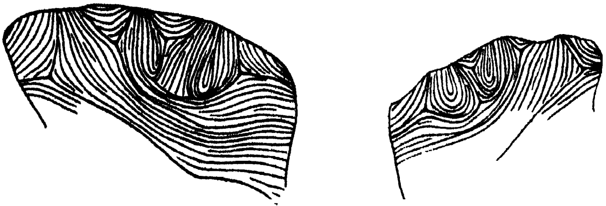
The particular significance of this set of prints is not alone the apparent inheritance of combinations of rare patterns and their vestiges with other rare patterns or with totally suppressed ones, but the extent and limitations of hereditary control and the indication of the method by which pattern modification may occur. It is obvious that it is not so much a *specific type of pattern* which is inherited as the *general type of pattern modification,* and that this is not a matter of undeviating progression from one generation to the next, since in this case three hands among the sons show a less advanced stage of degeneration than does that hand of the father from which the inheritance seems most definitely to have come. Of course, we do not know the configuration of the father's parents nor of his brothers and sisters, so that our data are meager, but suggestive at least of the fact that variation from one generation to the next in this matter of dermatoglyphics may move or fluctuate in either direction, and that the complete degeneration of the primitive typical whorl of considerable size to the open-field configuration must be a long and slow process, if dependent wholly upon fortuitous variations.

In this connection one should note also that it frequently happens, especially when the patterns have undergone extreme reduction while still retaining the primitive whorl type, as in the case of the fourth interdigital of the right hand of no. 1212 (pl. 3, B), a small amount of variation in the relationship of the ridges would place the pattern in one or another quite different type. This small amount of variation is beyond the limits of hereditary control, and it might thus happen that a wholly different morphological type of pattern would appear in offspring from that present in the parent and still be the result of inheritance from that parent. Obviously, as stated above, whatever it is that is inherited in the field of dermatoglyphics, it is not definite types of morphological patterns.

Cummins ('26) has shown quite conclusively from his study of configurations in abnormalities as well as from his study of the relation of pad elevations in the embryo (always more pronounced than in the adult) to the ridge configuration upon them, that the disposition of the ridges as they are formed is controlled by the shape of the area upon which they are disposed, and that, therefore, if there is an inheritance of configuration it is not a direct one, but rather one which results from the inheritance of the topography of the areas covered by the ridges. Evidence of the apparent inheritance of configurations is, however, not lacking (Wilder, '16), and it has been shown, moreover, that this inheritance seems to conform to mendelian principles; and yet it is quite obvious from all of the evidence presented that we have not yet discovered what the unit characters are with which we are dealing.

Although our discussion of pattern modification has dealt thus far mainly with patterns which are in process of reduction, in contrast to these, it occasionally happens that the third and fourth patterns show the influence of a spreading and flattening of the pad area, rather than its reduction. In such cases the boundaries of these patterns come into close proximity, and the identification of triradii in some cases becomes a purely arbitrary matter. This is especially true of the proximal triradii of the two patterns, which are identifiable only so long as both are present (fig. 17 a); but when there is only one, it might be interpreted as belonging to either pattern and is probably the result of the coalescence of the two proximal ones (fig. 17 b).

It is along the line of this extension of pattern areas through the flattening of the whole pad that we find the interpretation of such a unique configuration as no. 647 figured by Wilder ('16, p. 153) and formulated by him as 1.7.1.1 or 1.1.1.1, the actual prints being reproduced here as plate 7. Here, although at first sight the configuration seems to be that of open fields between main lines, all of which converge toward the center of the palm to follow the general course of the longitudinal flexion creases, there is in reality a single proximal triradius, the two distal radiants of which, though difficult to follow because of the fineness of the ridges and the interference of the transverse flexion creases and of the numerous minor wrinklings of the skin, can be traced, one into the third and the other into the fourth interdigital space. This proximal triradius may thus be interpreted as due to the coalescence of the proximal triradii of the two patterns. Its center lies so far proximally that it nearly coalesces also with the radial triradius of the hypothenar pattern. This interpretation is consistent with the first of the alternative formulations given by Wilder. Strangely enough, however, such is the convergence of ridges in this unique configuration that line C can with equal accuracy be traced to a termination in the carpal region.



17 a

17 b

Fig. 17 Tracings of distal palmar regions of two prints to show the effect of combinations of large third and fourth interdigital patterns. a, no. 7224, right, from the Carnegie Jamaican Collection of 'Browns,' with proximal triradii of both patterns present; b, no. 794, left, from the Wilder Collection, with probable coalescence of the proximal triradii of the two patterns.

One looks with interest to see whether in either of the two parents a similar configuration occurs, to find no similarity except in the hypothenar region of the mother. The configuration of both parents in the distal region of the palm is of quite the usual transverse type.

DISCUSSION AND CONCLUSIONS

In the manifold variations of configuration exhibited by the distal region of the human palm, one sees evidence of the working of two sets of forces, both obviously mechanical in nature, one conservative, the other progressive. The conservative one goes back in its antiquity to the development of the walking pads of primitive mammals; the progressive one, of later origin but also ancient, dates from the time when first the walking chiridium began to be used also for prehension, and it has been continually active and continually changing as in the evolution of the higher primates and man, prehension itself has become more varied in its adaptation to changing demands.

The conservative influence tends to hold the ridges to their more or less concentric arrangement with reference to the pad elevations, thus preserving the primitive patterns. The progressive influence tends to break down the boundaries of the patterns, preserving only those which chance to fall into systems of ridges parallel with the various lines of flexion, which are themselves changing with every slight difference in functional adaptation of the hand. With the advance of the progressive forces as the walking function of the hand has given way more and more to the prehensile one, has come the inevitable reduction of the pads themselves, both in elevation and in area, and the lessening thereby of the hold of the conservative influences, the patterns undergoing, as a result, characteristic degeneration modifications. Thus, in spite of the great variety of configurations presented in this distal region of the palm, we may see certain fundamental ways in which the conservative influences are giving way before the progressive. These influences are not, however, necessarily antagonistic in all respects, for any flexion of the palm which follows as a part of its course a portion of a pattern boundary tends naturally to preserve that boundary. Certain pattern boundaries may therefore be held to a more constant occurrence by this combination of the conservative and the progressive influences.

The disintegration of the distal boundaries of patterns, which seems usually to be the first step in their degeneration, may perhaps be attributed to the movement of abduction and adduction of the separate digits so characteristic of the human hand. Such movements introduce a minor longitudinal flexion crossing each interdigital area and tending to break down the distal boundary of the pattern. This would have the effect of preserving the distal divergent while driving the embracing radiants of the triradius in toward the core of the pattern, the end of this line of variation being inevitably the transformation of a whorl into a distally open loop.

Of the three interdigital patterns under consideration, the second has given way most completely, as shown by its rare occurrence, before the progressive influences. This is obviously due to the fact that the distal transverse flexion crease—a relatively late acquisition expressive of the simultaneous flexion of the last three digits—invades the second interdigital area, curving obliquely to a vague distal termination between the second and third digits and thus crossing the locus of the pattern. If the pattern is present, it lies usually wholly on the radial side of the crease and its component ridges tend to take a course either parallel with the crease or else parallel with the digital system of ridges which follow the course of the flexion of the index finger. The combination of these two flexion forces thus holds constant in occurrence a characteristic digital triradius at the base of the index finger, usually the only remnant of the pattern boundary. This triradius, as is indicated by the types of patterns which do occur, is either the radial or the proximal triradius of the pattern, or is the result of the fusion of the two, while the ulnar triradius has thus far in our experience never appeared. In by far the large majority of hands the whole second interdigital space is an open-field configuration parallel with the oblique course of this part of the distal transverse flexion fold. The main line, designated A, which forms the radial boundary of this area, shows, in the absence of a pattern, little variation in its course, which is in general proximal to the distal transverse flexion crease and at first parallel with it. It seldom crosses to its distal side, but often swerves proximally to follow the course of the more oblique or even that of the longitudinal flexion creases of the palm.

The curved course of the distal transverse flexion crease across the palm is one which, after leaving the second interdigital area, forms a proximal boundary of the third and fourth areas. Its influence upon these patterns is therefore different in nature from its influence upon the first, for although it crowds the pattern areas into a definitely delimited region, at the same time it tends to preserve their proximal boundaries. Lying, as they do, distal to the crease on the concave side of its curvature, they are not subjected to the disintegrating effect of its influence as is the second interdigital, the locus of which is actually crossed by the crease. This difference is seen in the much greater frequency of occurrence of patterns in these areas, particularly of the simple looped type.

A further influence of the flexion fold to preserve pattern boundaries may be seen at the two ends of its course where its presence tends, in much the same way as at the base of the index finger, to preserve a typical digital triradius at the bases of the ring and little fingers, the main lines from which, B and D, respectively, tend to fall into the general system of ridges parallel with the flexion crease when they do not curve distally to embrace a pattern. As to the homologies of these triradii B and D and their respective main lines, we have seen that B is constantly the radial one of the third pattern, while D has a range of morphological possibilities which includes all three of the triradii of the fourth pattern, although in the large majority of cases it is the ulnar one which is thus preserved. It is probable that in the cases of the rare types of the fourth pattern, the degeneration of which results in the radial triradius, alone or in fusion with the proximal one, becoming the permanent digital triradius D, it is owing to the influence of the distal transverse flexion crease that the pattern is always so small, since these patterns lie in the restricted area between the flexion crease and the main line.

The chief influence of this flexion crease upon the patterns which lie distal to it arises from the confinement of the patterns to a very limited region of the palm. Thus, not only do the main lines B and D, the usual extreme boundaries of the two pattern areas, seldom cross this flexion crease, but the proximal divergents of the patterns, when present at all as distinct from the main lines themselves, are also confined within narrow limits. Thus, in the case of the fourth interdigital, in which the proximal divergent is more often present than in the case of the third, it frequently has but a short independent course converging toward the neighboring main line, upon which it abuts or with which it fuses, while in other cases it is deflected toward the distal border and either becomes involved in the third interdigital pattern or may bend more sharply to reach the distal margin within the fourth interdigital space. In the small number of cases in which a proximal divergent of the third pattern is present, it usually shows similar deflecting influences, as has been pointed out already in describing types of the third pattern. These facts indicate the probable method by which the proximal divergents and triradii of these patterns, particularly in the case of the third, have come to be almost wholly lacking.

Further effects of the encroachment of the flexion forces upon the pattern areas are seen in the marked tendency which has been pointed out toward the crowding of the third pattern in the direction of the base of the ring finger, so that not only do very small patterns and vestiges of patterns occur in this region, but it not infrequently happens that digital triradius C, which is usually morphologically the ulnar triradius of the pattern, is wholly lacking through the suppression of main line C with the complete disappearance of the pattern. Even when this complete suppression of line C has not occurred, the abbreviated course of the line or its frequent sharp deflection into the fourth interdigital area bears witness to the limitation of space to which it is subject.

Such configurations as those shown in plate 1 where, in one instance (A) not only the third interdigital pattern itself, but also triradii B and C are obliterated, while in the other (B) a similar complete obliteration of the fourth pattern, including triradius D, has occurred, are examples of the complete triumph of the progressive forces over the conservative in the evolution of the dermatoglyphics of this region of the palm, although these two cases themselves obviously differ from each other in the type of prehensile use of the hand which they express.

In the course of this paper mention has been made of differences between right and left hands as well as to racial differences in the general trends exhibited in the evolution of dermatoglyphic configurations. These matters constitute the subject of another paper.

One cannot engage in any careful study of dermatoglyphics without becoming more and more impressed with the unlimited possibilities of this field for the solution of some of the most fundamental biological problems. In both their phylogenetic and ontogenetic origin the ridges themselves, made up as they are by the fusion of rows of separate units, each the bearer of a sweat pore, give every evidence of being the product of mechanical pressure and of serving a mechanical function, but always subject to slight individual variations, since each ridge in each individual is constituted anew from separate units, themselves variable in number and arrangement.

With the evolution of the prehensile from the walking chiridium there is every evidence that the changes in mechanical forces to which the ridge-covered areas were subjected have been accompanied by corresponding changes in ridge configuration of such a nature as to suggest response to mechanical forces to the end that the ridges may best subserve their mechanical function. But what is the biological mechanism by means of which these changes in configuration tend to keep pace with the changes in use of the chiridium?

We are dealing here with structures which are laid down during the last half of fetal life with the arrangement which they are to have throughout the life of the individual, and hence not themselves subject to modification after they are once formed, although, by use, the surfaces which they cover may be modified in their topography through changing pressure conditions. Moreover, granting a certain range of variation in the configurations which is not subject to hereditary control, there is much evidence that ridge configurations are inherited and, moreover, that this inheritance is in accordance with mendelian principles. The logical conclusion is, then, that it is through the germ cells that the progressive changes, adaptive in nature, have come about in the evolutionary process. As summed up so succinctly by H. H. Wilder ('16, p. 237):

> One could hardly find a more startling combination of principles . . . ., supporting at the same time the ideas of Lamarck, Mendel, and Weismann with Darwin left entirely out, since with the reduction in the size of the friction ridges and the increase in the size of the body, they can be of no real selective value above the Cercopithecidae.

Such is the enigma of dermatoglyphics!

LITERATURE CITED

CUMMINS, HAROLD 1926 Epidermal ridge configurations in developmental defects, with particular reference to the ontogenetic factors which condition ridge direction. Am. Jour. Anat., vol. 38.

CUMMINS, HAROLD, AND MIDLO, CHARLES 1926 Palmar and plantar epidermal ridge configurations (dermatoglyphics) in European Americans. Am. Jour. Phys. Anthrop., vol. 9.

CUMMINS, HAROLD; KEITH, H. H.; MIDLO, CHAS.; MONTGOMERY, ROBERT; WILDER, H. H.; AND WILDER, I. W. 1929 Revised methods of interpreting and formulating palmar dermatoglyphics. Am. Jour. Phys. Anthrop., vol. 12.

HEPBURN, DAVID 1895 The papillary ridges on the hands and feet of monkeys and men. Sci. Trans. of the Royal Dublin Soc., vol. 5, series II.

KEITH, HARRIET H. 1924 Racial differences in the papillary lines of the palm. Am. Jour. Phys. Anthrop., vol. 7.

MAIRS, G. T. 1925 An arrangement of friction-ridge dermatographs (chart). Finger Print Magazine, vol. 7, no. 3, pp. 16 and 17.

WHIPPLE, I. L. (MRS. H. H. WILDER) 1904 The ventral surface of the mammalian chiridium, with especial reference to the conditions found in man. Zeits. f. Morph. u. Anthrop., Bd. 7.

WILDER, HARRIS H. 1903 Palm and sole impressions and their use for purposes of personal identification. Pop. Sci. Monthly, Sept.

——— 1916 Palm and sole studies, I–VII. Biol. Bull., vol. 30.

——— 1922 Racial differences in palm and sole configuration. Palm and sole prints of Japanese and Chinese. Am. Jour. Phys. Anthrop., vol. 5.

——— 1925 Palm and sole studies. VIII. Occurrence of primitive patterns (whorls). Biol. Bull., vol. 49.

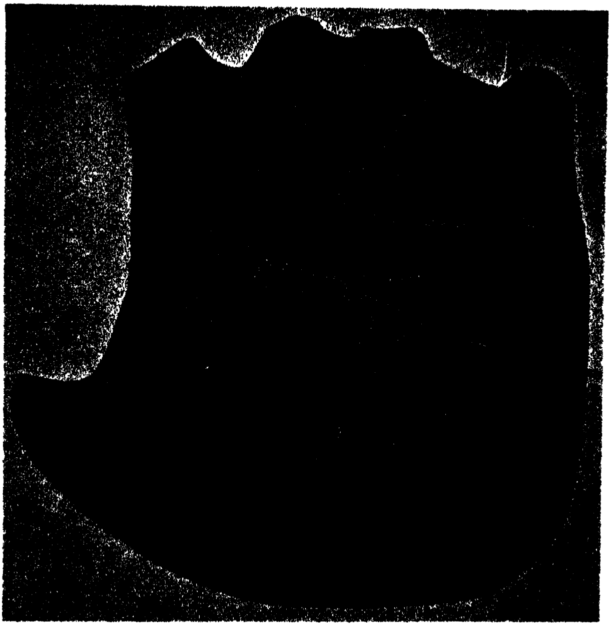
——— 1926 Palm and sole studies. IX. The morphology of the hypothenar of the hand; a study of the variation and degeneration of a typical pattern. Biol. Bull., vol. 50.

PLATE 1

EXPLANATION OF FIGURES

A Print of a right palm (Caucasian), showing the complete obliteration of the interdigital patterns, involving also the loss of digital triradii B and C. Note the transverse course of the ridges in the whole distal region of the palm.

B Print of left palm of a Bontoc Igorot from the Wilder Collection, no. 485, showing complete obliteration of the interdigital patterns, involving also the loss of digital triradius D. Note the general oblique course of the ridges crossing the distal region of the palm.



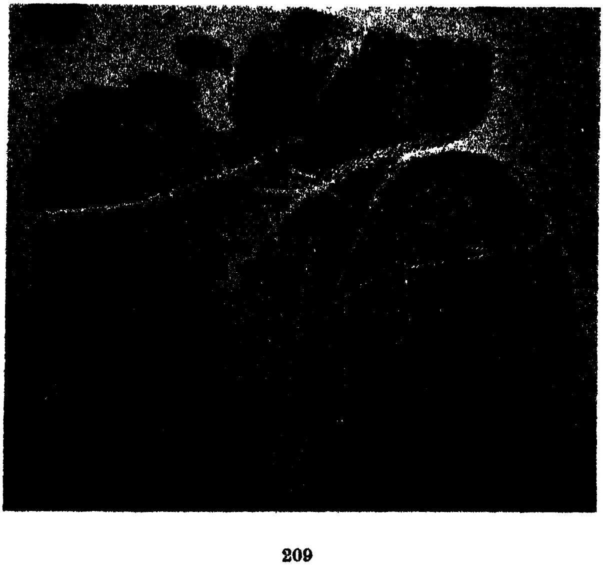


PLATE 2

EXPLANATION OF FIGURE

Palm prints of no. 1211, Wilder Collection, brother of nos. 1212 and 1213, showing unusual configurations.

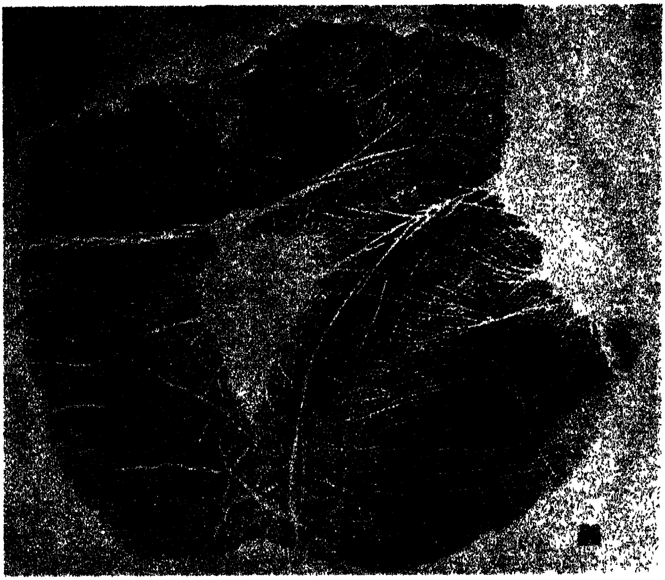
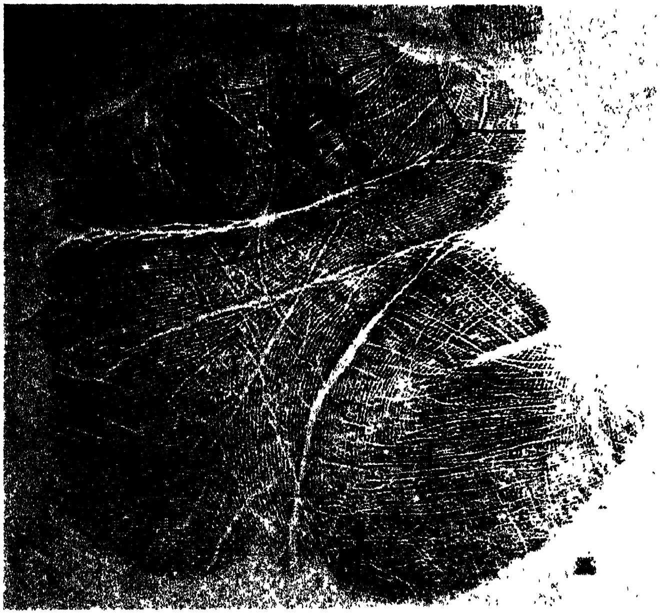


PLATE 3

EXPLANATION OF FIGURE

Palm prints of no. 1212, Wilder Collection, brother of nos. 1211 and 1213, showing unusual configurations.



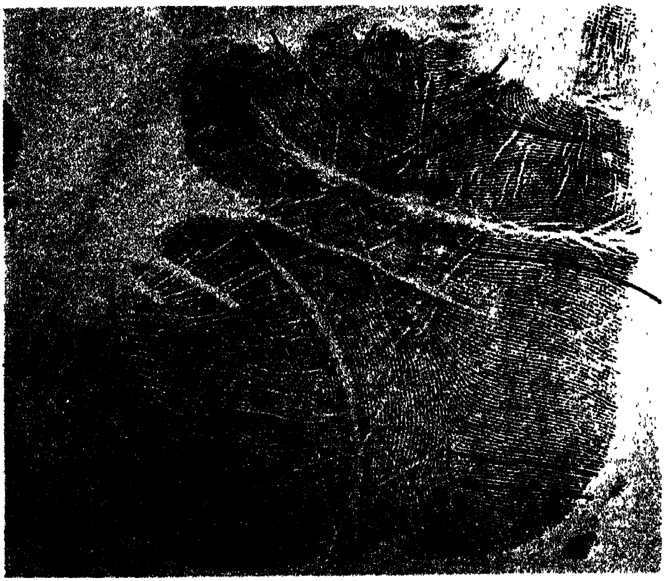


PLATE 4

EXPLANATION OF FIGURE

Palm prints of no. 1213, Wilder Collection, brother of nos. 1211 and 1212, showing unusual configurations.

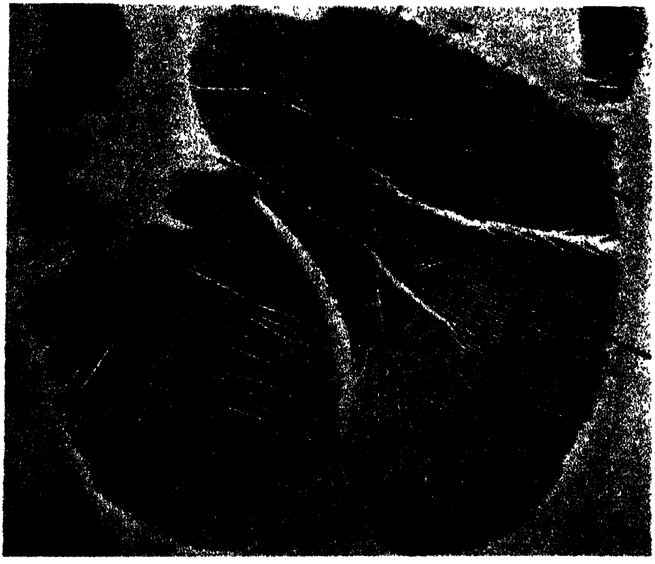
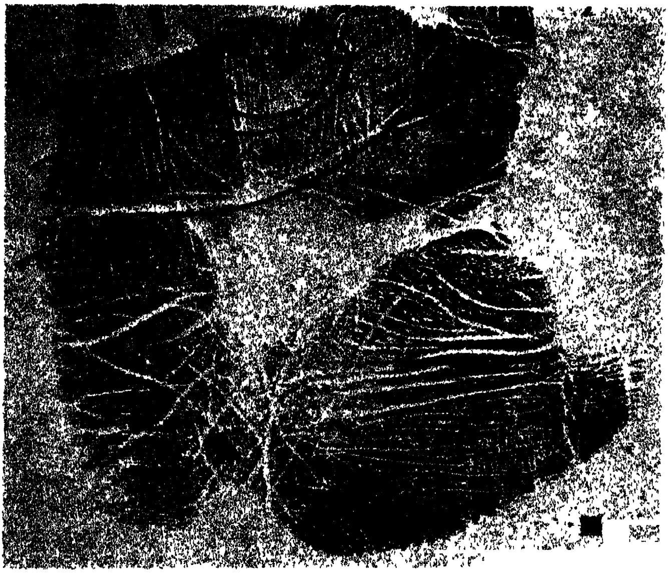


PLATE 5

EXPLANATION OF FIGURE

Palm prints of no. 1209, Wilder Collection, father of nos. 1211, 1212, and 1213.



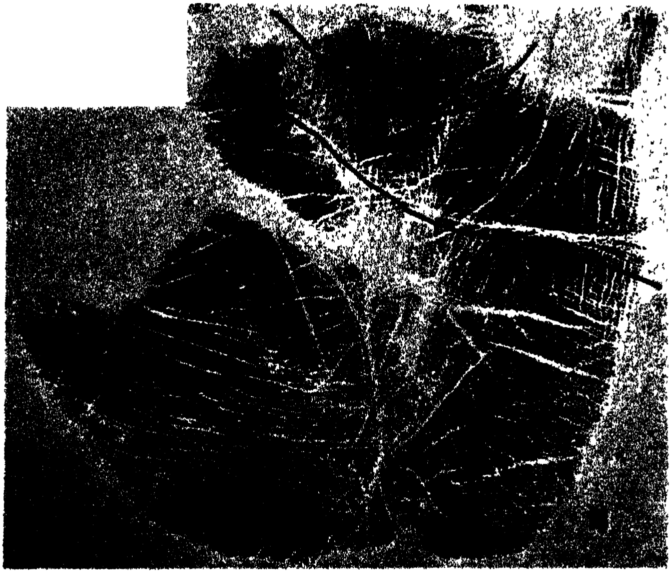
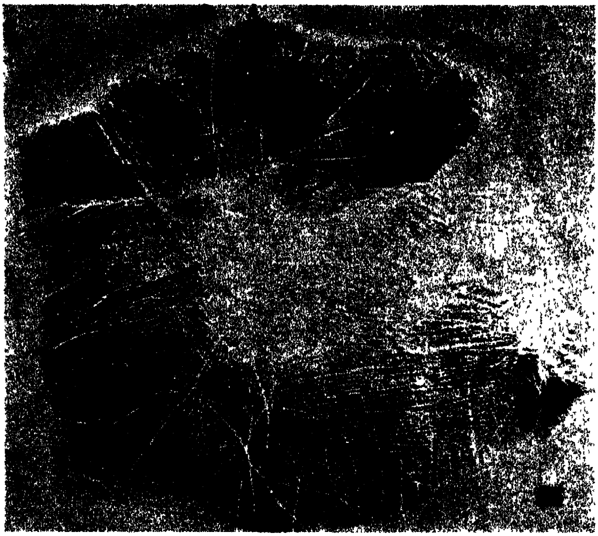


PLATE 6

EXPLANATION OF FIGURE

Palm prints of no. 1210, Wilder Collection, mother of nos. 1211, 1212, and 1213.



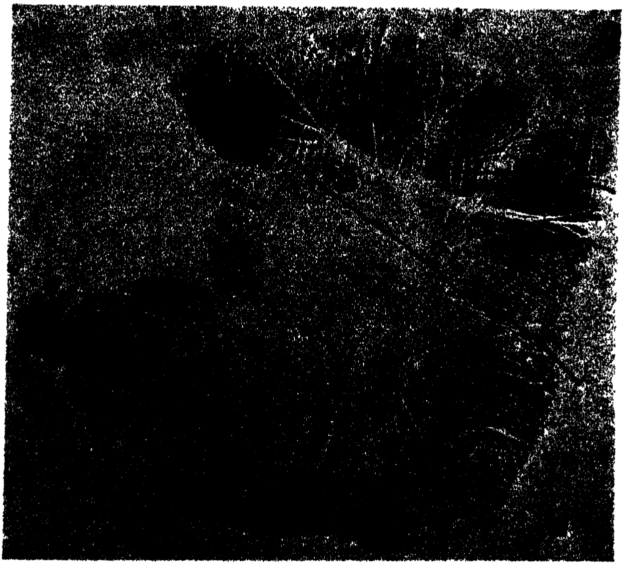
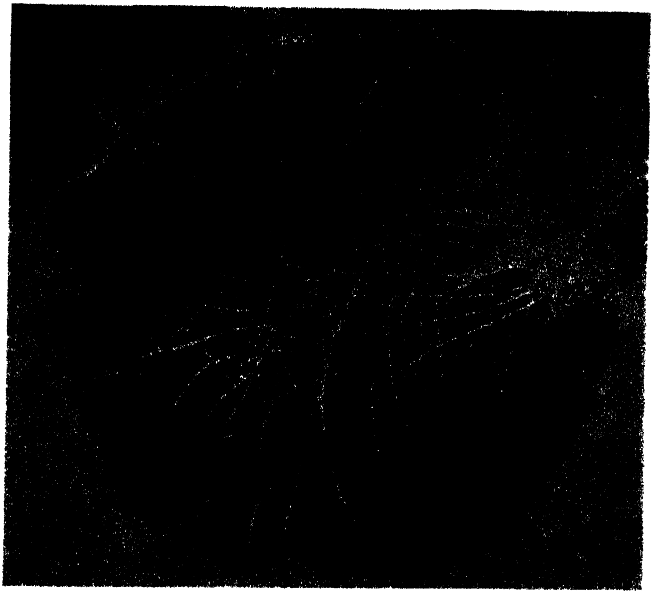
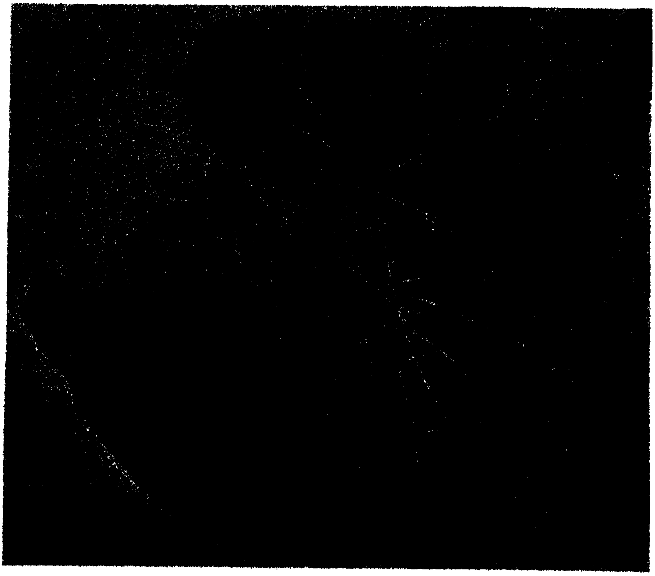


PLATE 7

EXPLANATION OF FIGURE

Palm prints of no. 647, Wilder Collection, showing a unique case of a general longitudinal direction of ridges of the whole palmar area, combined with a coalescence of third and fourth interdigital patterns.





A CONNECTING LINK BETWEEN THE ANNELIDA AND THE ECHIUROIDEA (GEPHYREA ARMATA)

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TWO TEXT FIGURES AND THREE PLATES (THIRTY FIGURES)

AUTHOR'S ABSTRACT

During the course of a hydrobiological survey of Monterey Bay, California, a series of pelagic organisms was taken at a depth of approximately 350 M., which appears to be a link connecting the Echiuroidea (Gephyrea armata) on the one hand and the polychaetes on the other. The range in body length is from 5 mm. to 27 mm.; and in an extended condition the anterior end of the body, with its palps and cirri, bears a close resemblance to a nereid. The supra-oesophageal ganglia and the ventral nerve cord, with eleven pairs of ganglia, are also strikingly annelidan in character. On the other hand, the spacious coelom, communicating with the exterior by a pair of anterior nephridia, allies it with the gephyreans. The same is likewise true of the gonad, which arises from the coelomic epithelium surrounding the genital vessel. The various systems are described in detail, and their resemblances to those of other annelids are indicated. This new species, Poeobius meseres, is made the representative of a new family, the Poeobiidae, which, provisionally at least, is included in the Echiuroidea.

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INTRODUCTION

Since the days of the pioneer work of Quatrefages ('47), and more particularly that of Hatschek ('80) on Echiurus, the theory that the Gephyrea armata constitute a division of the Chaetopoda has been gradually strengthened by the subsequent work of numerous authors. We now know that not only are setae present in the first-named group, but their mode of origin and musculature present a fundamental resemblance to that of the chaetopods. The nervous system also is of the typical annelid type, so far at least as its general relations are concerned. Furthermore, a study of the development of several species of this class discloses a distinct metamerism in the larvae in which the coelom and nervous system follow broadly the path of development characteristic of the typical polychaete. In brief, the problems now demanding solution are not so much concerned with the reality of this broader kinship, but rather with the interrelationships of the species involved and their evolutionary history.

An interesting side light is thrown on some of these questions by the discovery of a new species of animal which appears to hold a more or less intermediate position between the Polychaeta and the Echiuroidea. The appendages of the head, for example, and the metamerically segmented nervous system are distinctly annelidan, while the body wall, coelom, circulatory system, and gonad also strongly suggest gephyrean relationships. Still other features are of a more puzzling nature, and may represent special adaptations to the free-swimming existence characterizing this species. However, a discussion of these broader questions, including the systematic relationship of this new species, is best reserved for later paragraphs after the various systems of organs have been described in detail.

MATERIAL AND METHODS

During the summer of 1928, a preliminary hydrographic survey of Monterey Bay, California, was made under the joint auspices of the State Fish and Game Commission and the Hopkins Marine Station of Stanford University, with Dr. H. B. Biglow in charge. During one of the plankton hauls, made in July at the entrance to the bay at a depth of approximately 350 meters, a number of elongated free-swimming organisms were taken which Doctor Biglow kindly passed on to me for identification. On shipboard most of the specimens had received rough treatment, and had been preserved in formaldehyde; but in a living condition they were stated to be "as transparent as sagittas with the exception of the digestive tract, greenish in color." Three individuals were uninjured, and even the worst-damaged possessed intact organs, especially at the extremities of the body. Some of this material was stained in Delafield's haematoxylin, and was studied either as whole mounts or in serial section. One specimen was carefully dissected, and certain organs were compared with those of other individuals reconstructed from sections. In all, eleven specimens were captured, and, generally speaking, the preservation is good.

On the 19th of February, 1929, Dr. Tage Skogsberg, continuing the survey, brought in seventeen additional specimens. These had been taken from the same general locality as the first and at approximately the same depth. All were badly mutilated, but in two specimens the head was extended (fig. 5), and presents a far clearer appearance of this part of the body than had been gained from the dissection and reconstruction of sections of contracted animals.

EXTERNAL FEATURES

The smallest specimen measured 5 mm. in length, while the largest was 27 mm. long. In these two extremes, however, and in the case of the intermediate-sized individuals, the bodily shape and the configuration of the various systems of organs remained fundamentally the same, the only essential difference relating to the gonad, which was more or less undeveloped in the smaller animals. In lateral view (fig. 1) the body presents the appearance of a greatly elongated ellipse with the extremities of the major diameter somewhat truncated. In cross-section the outline of the body is moderately biconvex, the transverse and vertical axes throughout the length of the animal having an approximate ratio of 1:2 (fig. A). In the head region this lateral compression gradually increases until the extreme end of the body becomes developed into a cutwater of almost knifelike sharpness.

This paper was virtually completed before these later specimens were brought in, and a few of the drawings here reproduced were made with the belief that all of the tentacles were modifications of the prostomium and arose from practically the same level. The expanded specimens, noted above (figs. 5, 6, 7), however, clearly prove this to be an incorrect assumption. As may be seen in the figures, the 'tentacles' belong to two distinctly different groups. The first pair appears unquestionably to be prostomial, possibly homologous to the palps in certain polychaetes. They evidently are not to be looked upon as modified lips, which in this case completely surround the mouth, and are distinctly separated from the anterior pair of processes by a definite furrow (fig.

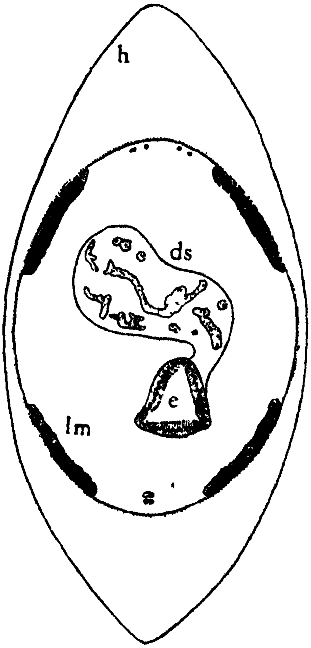

Fig. A Diagram of a cross-section of the body at the level of the gastric sinus, *ds*; *e*, gut; *h*, hypodermis and cuticle; *lm*, longitudinal somatic muscles.

12). Furthermore, each is traversed by a heavily ciliated groove, which stops short of the lips proper. During the feeding process it may be assumed that the mouth is more widely open than in the drawings, and the lips presumably are more closely pressed against the prostomial processes, so that food swept along the groove is readily brought within reach of the inwardly directed current produced by the heavy cilia lining the buccal cavity.

The second set of processes or cirri comprises five pairs symmetrically attached to the sides of the head a short distance posterior to the prostomial tentacles. Their arrangement thus corresponds closely to that in certain nereids, with which there is a strong presumption they are homologous. It is to be noted, however, that there are five pairs arranged in two ranks, two being in the dorsal one. Also, there is no clear line of demarcation between the pro- and peristomal regions. And, finally, in none of the series of sections is it possible to trace the innervation of these organs, although it is worthy of note that the first ventral ganglion represents in reality two fused ganglion pairs, as in the nereids.

As is to be expected, all of these appendages comprise the same elements as the body wall—an outer epidermal layer, and a delicate sheath of circular muscles resting upon a more powerful set of longitudinal fibers (fig. 24). The epithelial elements are more or less cuboidal structures bearing a heavy coat of cilia, while the muscle fibers are more compact than those of the body wall—a condition possibly correlated with active food collecting. Each tentacle contains a comparatively spacious schizocele traversed by blood vessels, which, with the aid of the circular muscles, probably aid in the extension of these organs. It may be added that the cilia on the appendages of the head appear to be the sole means of locomotion.

INTERNAL ORGANIZATION

Body wall

The body wall externally consists of a delicate cuticle beneath which, at succeeding levels, are located the hypodermis and the circular and longitudinal muscles, the last-named being separated from the coelomic cavity by the usual epithelium. It is thus seen that the tissues comprising the body wall of annelids generally are here represented, but, as will be described presently, the cuticle and hypodermis, throughout the length of the body, are separated from the underlying muscles by a perfectly normal fluid-filled space.

The cuticle appears to be a structureless, smooth, and very thin membrane demanding no further comment. The hypodermal layer measures 0.009 mm. in thickness on the average, and of the cells composing it all that need be said of the material in hand is that the nuclei are seemingly homogeneous bodies spaced at relatively wide intervals (fig. 19). No sign of cell boundaries has been discovered at any point.

In a strong light, with low magnification, the surface of the hypodermal layer is seen to be dotted with a multitude of minute white specks. More highly magnified, these become resolved into from fourteen to twenty-three nuclei (fig. 19), about one-half the diameter of the usual hypodermal elements, which also are without visible cell boundaries. In many instances a slender nerve bundle passes through the group, and where it skirts the organ the delicate fibers may be seen in many instances to enter its substance. It should be added that these nerves form, over the hypodermis generally, an extensive plexus in which at least one pair of nerves from each of the ventral ganglia plays a part.

Attention has been called to the fact that, although the customary layers form the body wall, there is a wide space between the hypodermis and the underlying musculature (fig. A). This cavity is filled with an apparently non-coagulable fluid, devoid of cells, which with the coelomic fluid gives to the body about the same turgidity as that of a medusa of the same size. Furthermore, the partial dissection of two specimens disclosed the presence of delicate connective-tissue strands extending from the somatic musculature to the hypodermis. Both of these animals were somewhat mutilated, but the greater number of trabeculae appeared to exist in the midregion of the body, and to a somewhat less extent in the neighborhood of the head and cloacal region. Elsewhere the hypodermal layer appears to be nearly if not entirely free. It is evident that this excessive schizocele development is a flotation device, increasing the bulk of the body without adding materially to its weight.

The somatic musculature comprises four great longitudinal bands, extending throughout the length of the body, and a more delicate external sheath of circular fibers. As may be seen in the figure (fig. A), each of the longitudinal bundles is separated by a relatively wide space, ventrally, dorsally, and laterally, the position of the muscles thus corresponding to the excessively developed dorsal and ventral longitudinal muscles of typical polychaetes. As is to be expected, these bands are widest and thickest in the middle of the body, and gradually diminish in size as the extremities are approached. The circular muscles form a relatively thin layer composed, as in the case also of the longitudinal system, of slender cells at least 0.4 mm. in length. These are in close contact in the longitudinal bundles, whereas the elements composing the circular muscles are separated at fairly definite intervals by comparatively wide spaces (fig. 26).

In all of the smaller specimens a membrane is attached to the outer surface of the circular muscle sheath. It is excessively thin, and both in whole mounts and in sections appears to be structureless with comparatively large nuclei widely spaced. In the larger individuals this same structure is present, but except at the extremities of the body it is separated by a distinct space from the somatic musculature. In figure 26, for example, which represents a portion of the body wall in the midsection of the animal, this space (lying between *bm* and *ce*) is 0.017 mm. wide, and in a perfectly regular and seemingly normal fashion gradually narrows as the ends of the body are approached. Its function is unknown, but the thought suggests itself that it is a basement membrane which normally is in contact with the hypodermis in other animals.

Coelom

The coelom extends throughout the length of the animal from the posterior borders of what may be termed the buccal mass to the rectum, and laterally is bounded by the somatic musculature. While it is reasonable to presume that in early stages there were mesenteries and septa, these have disappeared almost if not completely in the specimens in hand. The digestive tract, passing through it, is sheathed in coelomic epithelium, and evidently a similar coat covers at least a few of the blood vessels. A similar covering also appears to exist over the ventral nervous system, which also lies freely in the coelom, but the preservation of the material renders this somewhat uncertain. Anteriorly, protractors and retractors of the head span the cavity in attaching to the body wall, and posteriorly a few delicate connective-tissue fibers form a similar attachment for the rectum. The digestive tract and gonad likewise are anchored by a few very tenuous trabeculae, but that these are sheathed in coelomic epithelium is doubtful. In conclusion, it may be said that in the largest (type) specimen a few small clots float in the coelomic cavity, but whether these contain cells is not known. No coelomic corpuscles have been seen in sections. A pair of nephridia place the coelom in communication with the exterior.

Digestive tract

In the fully expanded specimens (figs. 5, 6, 7) the mouth is subterminal, being overarched by lips which are relatively thin dorsally, much thickened laterally, and are almost entirely lacking in the midventral line. The buccal cavity thus formed is subject, however, to wide differences in shape, since in several of the contracted specimens it becomes relatively long and tubular. The cells bounding its surface appear to be ciliated throughout, although those of the midventral line and borne on cuboidal elements are comparatively weak. Over the dorsal surface the cells are more columnar, the cilia heavier, while laterally the walls become distinctly thicker and are provided with a dense ciliated coat. Movements of this portion of the digestive tract are probably carried on by muscles in the buccal wall, and possibly by vessels which likewise are embedded in its substance.

At the posterior termination of the snout or proboscis, in a ventral position, is a comparatively wide dilatation of the buccal or pharyngeal cavity that may be termed the buccal pouch (figs. 2, 6, *p*). The heavy ciliated epithelium of the buccal cavity extends into this dilatation, forming its lateral walls. Its anterior wall is a continuation of the low epithelial lining of the ventral side of the proboscis, while its posterior surface consists of a pad of cells whose appearance and possible function call for a brief description. This so-called pad consists of two arc-like masses, separated from each other by a relatively thin layer of connective tissue, which curve about the posterior border of the buccal pouch. Internally, they are covered with a sheet of low epithelial cells (fig. 20), and externally by a muscle sheath whose more exact relations will be described presently. The pad itself consists either of loose connective tissue or, what is more probable, of cells, separated by slender strands of connective tissue, each of which is largely occupied by a liquid-filled vacuole. At all events, when the structure was removed in the course of dissection, it proved to be a relatively firm elastic organ.

Attached as it is to the posterior walls of the buccal pouch, it appears probable that the pad with its elastic properties is a device for permanently keeping the pouch in an open distended condition. In such a state it receives the detritus, diatoms, and other microscopic organisms forming this animal's food supply. When the pouch becomes filled, two distinct operations evidently ensue. The first consists of the closure of the opening from the proboscis to the pouch. This is effected by the contraction of a species of sphincter muscle (fig. 2), which as a transverse band is attached at the junction of the pouch and proboscis. After communication with the proboscis has been cut off, the cavity of the pouch is narrowed by the contraction of muscle fibers which form a loose sheath over the pouch generally. This results in driving the food into the wide dilatation, or crop, lying immediately posterior to the buccal mass.

The walls of the crop in immediate contact with the proboscis are composed of high columnar ciliated cells charged with a highly vacuolated secretion. Abruptly these change more posteriorly to cuboidal and low columnar elements, also ciliated, in which the protoplasm is relatively dense. This state of affairs continues to the first loop of the gut, where the typical oesophageal cells gradually merge with others of twice their height (fig. 27) and containing relatively large amounts of some vacuolated secretion. If cells of this character are actually to be considered gastric, and there appears to be no valid objection to such a view, the stomach then comprises the coiled smooth-walled section of relatively large caliber shown in figure 1. Large quantities of food materials mask the ciliated coat in large measure, but in the most favorable material it appears that a ciliated tract extends along one side of the stomach from the oesophagus to the intestine, where it becomes continuous with a typhlosole-like structure (fig. 23) continuing to the rectum. It is worthy of note that this 'typhlosole' corresponds in position and structure to the ciliated groove in the intestine of the Echiuridae. As may be seen in the figure, the cells of the intestine in general are low cuboidal elements, while those of the groove are of greater height and are composed in large measure of vacuolated protoplasm, evidently glandular. All of the cells are ciliated (lacking in the figure), those of the groove being relatively longer.

What may be termed the anal gland is a comparatively large, paired, somewhat lobulated organ extending forward on each side of the rectum (fig. 16) and posteriorly opening ventrally into the intestine immediately in front of the anal opening. It has no communication with the coelom. Anteriorly, where the active cells are relatively few, this structure is plainly a hollow pouch with thin walls and indefinite cell boundaries to which are attached scattered spherical gland cells with an average diameter of 0.04 mm. These are loaded with a vacuolated secretion staining intensely in haematoxylin. More posteriorly, the gland becomes more compact, the cells and secretion practically filling the lumen. Its function is unknown.

The dorsal longitudinal bands of the somatic musculature, in the posterior end of the body, assume a more lateral position and each splits into two divisions. Of these the more dorsal pair becomes inserted into the ventral wall of the rectum immediately dorsal to the outlet of the anal gland. The more ventral pair is attached to the lateral walls of the gut opposite to the opening of the anal gland. Each of the ventral somatic longitudinal muscles also forms two subdivisions. The more dorsal pair passes between the halves of the anal gland and is inserted in the ventral surface of the rectum immediately in front of the duct. The ventral pair continues to a more posterior position, and branching, is inserted in the ventral wall of the rectum.

Circulatory system

The circulatory system is completely closed, and as most of the main vessels traverse the coelomic cavity, their course is accordingly readily followed. In the extremities of the body, on the other hand, the circulation of the blood is most difficult to follow. Posteriorly, the main branches have been traced in dissections and by the reconstruction of sections, and accordingly their relations are fairly clear. In the region of the tentacles contracted vessels with a tortuous course and frequent branching have rendered it impossible to gain a clear picture of their distribution, despite an extended study of dissections, whole mounts, and sections. Nevertheless, the main features of the blood system are fairly clear, and after these have been described an attempt will be made to trace the general circulation.

When compared with typical annelids, it appears that the circulatory system in the present species is least modified in the posterior half of the body. Here there is a well-marked dorsal vessel (fig. B, *C*), a ventral vessel extending above the ventral nerve chain throughout the length of the body, and various connecting branches. The dorsal vessel, spanning the coelomic cavity close to the middorsal line about midway between the gut and body wall, extends from the region of the rectum to the level of the forward border of the stomach or first coil of the digestive tract. Throughout its course it develops five commissural branches, which, adhering to the somatic musculature, ultimately unite with the ventral vessel. More posteriorly, the main trunk breaks up into a system of capillaries which finally become lost to sight in the tissues of the rectal region. At the level of the posterior end of the oesophagus this dorsal vessel gives rise to four comparatively small vessels, which, extending anteriorly, soon become arranged in pairs on each side of the midline of the body. Furthermore, they usually become early attached to the somatic musculature, and in this position continue into the anterior end of the body. The outermost branch (fig. B, *A*, *o*) on each side, when opposite to the posterior limits of the buccal mass, bends abruptly ventrally and unites with the ventral vessel. The more median pair continues its course beyond this point, and finally becomes lost to view in the tissues of the proboscis sheath. In one specimen there are indications that these last-named twigs communicate with branches of the small extension of the ventral vessel which makes its way to the region of the first ventral ganglia, but positive proof on this point is lacking.

After the development of these four branches, the dorsal vessel almost immediately expands into a relatively huge chamber (fig. B, *B*, *ds*), unique in several particulars. Its walls, in the first place, are little if any thicker than those of an ordinary vessel, it invariably is gorged with blood, it

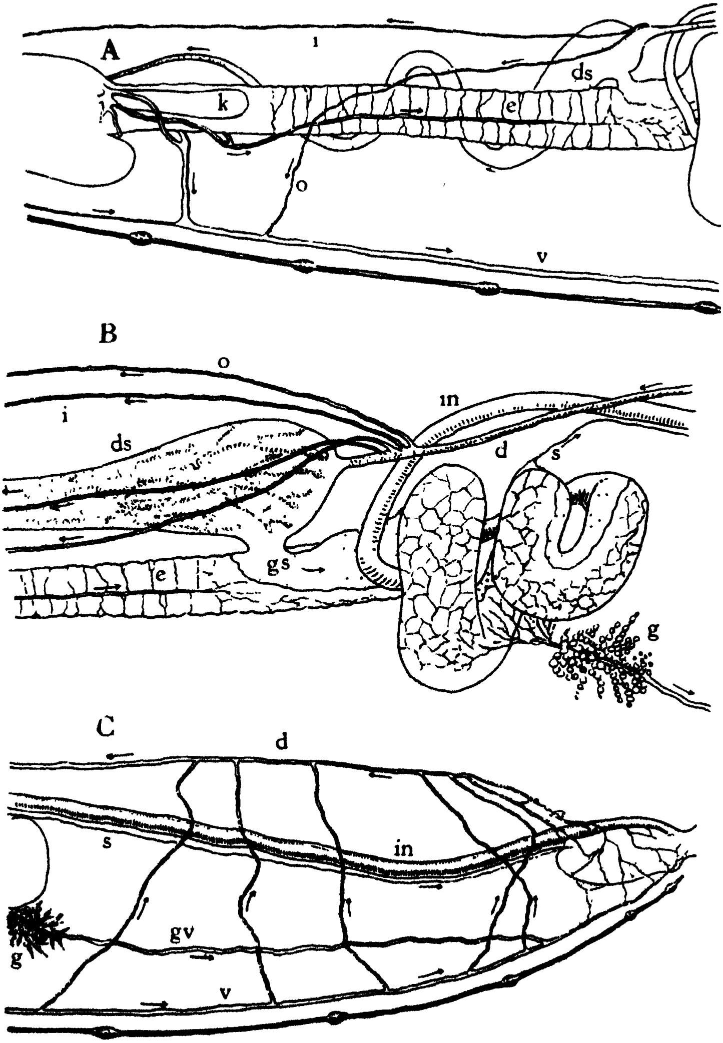

Fig. B Diagram illustrating the circulatory system and the probable course of the circulation in (A) the anterior; (B) middle, and (C) posterior regions of the body. *d*, dorsal vessel; *ds*, dorsal sinus; *e*, oesophagus; *g*, gonad; *gs*, gastric sinus; *gv*, genital vessel; *i*, inner branch of dorsal vessel; *in*, intestine; *k*, nephridium; *o*, outer branch of dorsal vessel; *s*, subintestinal vessel; *v*, ventral vessel.

contains masses of cells which will be described presently, and its relations prove it to be an undoubted section of the circulatory system. Anteriorly, this vessel is thrown into a series of loose loops, and, lying freely in the coelom and gradually diminishing in caliber until it is scarcely larger than the dorsal vessel in the posterior half of the body, it enters the head. For the sake of brevity, it may be termed the dorsal sinus.

No trace of definite corpuscles has been found in any of the usual blood vessels, and preserved material gives no clue as to whether haemoglobin is dissolved in the plasma, as in Sternapsis, or whether it is colorless, as in Thalassema. In the dorsal sinus, on the other hand, there are numerous cells which present an unusual appearance. For the sake of clearness, it is necessary to state that the walls of this organ, as in the case of the blood vessels generally, are composed of excessively thin cells with darkly staining nuclei. No muscle fibers have been discovered, nor is there here apparently any coelomic epithelial sheath. In its anterior extremity, at the point where it divides to connect with the buccal mass, the cells of its walls become relatively numerous and appear to be in an active state of proliferation. Certain it is that at this point four symmetrically placed cell columns (fig. 15, *b*) are attached to the inner surface of the walls of the dorsal sinus, and, suspended freely in the lumen, extend posteriorly. Evidently cell division continues beyond the point of attachment, since each of these columns gradually increases in diameter and finally forms several branches (see cross-section, fig. 15, *c*), whose tips extend to the end of the sinus in the middle of the body. Each column thus resembles a tree attached by the base of the main trunk, but otherwise free and completely bathed in blood.

At and near the point of their first development the cells of each of these columns appear to be non-vacuolated, but in several specimens all assume the appearance represented in figure 9, where a great vacuole fills the major portion of the cell. In this condition they continue to the free extremities of each branch. At no point are there any signs of disintegration nor of separation of individual cells from the main mass. No reasonable explanation suggests itself as to the function of this structure.

The posterior extremity of the dorsal sinus is in contact with the posterior end of the oesophagus (figs. A, B), where it develops an extensive sinus, applied to one side of the gut, which in one especially favorable specimen was followed throughout the entire length of the stomach. It is probable that it communicates directly with the meshwork of vessels attached to the walls of the digestive tract generally, but actually no such connections have been traced.

The ventral vessel, as is indicated in the figures, is a relatively large trunk, dorsal to the ventral nerve cord, and extending from the rectum to the buccal mass. Posterior to the last commissural branch it continues as a slender canal, which upon branching becomes lost in the rectal tissues, as do the subdivisions of the dorsal vessel with which in all probability they communicate. Anteriorly, the ventral vessel breaks up into two main divisions, a delicate median one, extending as far forward at least as the anterior end of the ventral nerve chain, and a much larger one, which, proceeding dorsally, divides on the under surface of the oesophagus before entering the buccal mass. Their subsequent course is described in connection with the circulation of the buccal mass (p. 239).

About the level of the anterior end of the rectum the ventral vessel communicates with a branch of about equal size, which may be termed the genital vessel (fig. C, *gv*). This last-named division courses anteriorly, dorsal to the ventral vessel, yet ventral to the intestine, and passes into the gonad. Here it breaks up into a multitude of capillaries which extend in different directions. In young individuals, where these smaller branches are not buried in a mass of germ cells, it has been possible to trace them at several points into the plexus of vessels covering the stomach wall (fig. 14), and also in one case a connection was noted with the small vessel extending along the ventral surface of the intestine (fig. B, *A*, *s*).

Turning now to the circulation on the walls of the digestive tract, that of the oesophagus will first be considered. In figure 4 two vessels are shown which, leaving the head region, unite in the midventral line to form a single canal (*ev*). Coursing posteriorly, this vessel soon assumes a lateral position, and closely applied to the left side of the gut can be followed to a point adjacent to the stomach, where it breaks up into a system of capillaries (fig. B, *B*). About the level of the posterior end of the kidney in figure 4 a similar vessel, faint at first but soon assuming the same diameter as the left lateral one, can also be traced to a point close to the stomach, where it also forms a plexus. These two lateral vessels are united by frequent commissural branches passing both dorsally and ventrally around the oesophagus. There are indications, not perfectly clear, however, that the plexus formed at the posterior termination of the lateral oesophageal vessels is continuous with that coursing over the stomach. Also attention has been called to an extensive gastric sinus, a branch of the dorsal sinus, which also may connect with the gastric plexus. Certain it is that the stomach is provided with an extensive blood system, and it is fairly certain that it is drained off by means of the genital vessel, a small portion only leaving by way of the subintestinal branch.

From the above description it will be seen that the head region is supplied by three vessels, the ventral, the oesophageal, and the dorsal sinus. The ventral vessel (fig. 4, *v*) leaves the region of the ventral nerve cord midway between the first and second ganglionic masses, and passing dorsally, comes in contact with the under surface of the oesophagus. Here it divides, and each branch, after extending anteriorly and laterally, skirts the outer surface of the kidney and enters the tissues of the head. The left oesophageal vessel, a short distance posterior to the head region, also divides in the midline, and each of the resulting branches runs a course nearly parallel to the subdivisions of the ventral vessel with which it unites at the anterior border of the kidney. The subsequent distribution of these vessels could not be determined with perfect certainty. There are indications that they supply the cirri, tentacles, and snout, and during the process become united both dorsally and ventrally across the midline.

The distribution of the dorsal sinus in the head tissues also remains an unsolved problem. In one specimen especially (fig. 8) this vessel, upon reaching the region of the brain, was seen to divide, and each division was traced to the anterior border of the kidney. Sections of several individuals indicate that the branches subsequently developed also, also enter the tissues of the tentacles, snout, and cirri, but their exact route could not be traced.

The relations of these various elements of the circulatory system may perhaps become somewhat clearer after an attempt has been made to trace the course of the blood throughout the body (fig. B). At the outset it may be assumed that, as in annelids, the flow through the dorsal vessel and dorsal sinus is in an anterior direction. Accordingly, the blood in one (*o*) of the two pairs of branches, originating from the dorsal vessel proper at its junction with the dorsal sinus, flows into the ventral vessel, while the other (*i*) also streams anteriorly and may unite with a small branch of the ventral vessel in the region of the suboesophageal ganglion. Blood also flows from the dorsal sinus over the walls of the stomach and anteriorly courses through the tissues of the head. The current leaves the head by two channels—the oesophageal system and the main portion of the ventral vessel. The first-named forms a plexus, which spreads over the oesophagus and probably the stomach, and together with the gastric sinus, originating from the dorsal sinus, drains into the ventral vessel by way of the genital branch. A very small portion is also directed into the cloacal region through the subintestinal branch. In the commissures, uniting the ventral and dorsal vessels, the flow is dorsalward, which is likewise true of several branches in the tissues of the rectum and anal gland.

Nephridia

The nephridia, two in number, are situated in the anterior end of the coelomic cavity, which in whole mounts of expanded specimens was found to extend forward to the base of the cirri. Each presents the form of a U-shaped tube, with the inner and outer openings in relatively close contact. The inner opening, the nephrostome, communicating with the extreme anterior end of the coelom, is borne on the summit of a slender tube (fig. 2, *n*) composed of cuboidal, slightly vacuolated cells. This section more posteriorly rapidly expands into the main body of the nephridium, which, after extending posteriorly a variable distance, bends sharply upon itself, and, proceeding forward, reaches the level of the inner opening. Here the canal narrows, and sections show that it communicates with the exterior by a narrow pore on the side of the body immediately behind the cirri. The cells of the nephridia are highly vacuolated elements whose general appearance is sufficiently represented in the figures (especially figs. 21, 22).

Gonad

The genital vessel in the region of the stomach, or coiled portion of the gut, breaks up into a system of capillaries, which, as noted previously, become continuous with the gastric plexus. In this general locality one frequently notes the presence of distinct rounded cells, with compact nuclei, attached to the walls of the capillaries (figs. 14, 17). At other points, chiefly in older individuals, other groups of cells appear, of smaller size and more compact appearance (fig. 17), also attached to the walls of the smaller vessels. It may be assumed, in the light of what we know of the development of the germ cells in related species, that these are modified elements of the coelomic epithelium becoming transformed into germ cells. In the case of the females, which preponderate in the material in hand, the gonad becomes a conspicuous feature, measuring fully 2.0 mm. in transverse diameter. The ova, still attached, measure from .09 to 0.1 mm. in diameter, and from their rounded appearance are practically mature. The testis, on the other hand, has not been found to exceed a diameter of over 0.45 mm. even in specimens with a length close to 20 mm. No germ cells in either sex have been found floating in the coelom.

Nervous system

The nervous system conforms to the usual annelid type with supra-oesophageal ganglion, circumoesophageal connectives, and a ventral nerve chain comprising eleven pairs of ganglia with the usual commissures and connectives. In the more fully expanded individuals the brain is seen to occupy a position about midway between the tentacles and cirri (fig. 5). It is a definitely paired organ, as are the nerves leaving its dorsal and lateral borders, but the destination of these nerves is unknown, since almost immediately they plunge into the dense tissues of the head and cannot be differentiated. The connectives to the ventral cord are in large measure attached to the inner surface of the somatic musculature, and in one especially favorable specimen each was found to develop a clearly defined ganglion (fig. 10) before entering the suboesophageal ganglion. This last-named structure is clearly seen, both in whole mounts and in sections, to consist of two pairs of ganglia separated by a slight groove as in some of the annelids (fig. 10). The nerves to which they give rise supply the somatic musculature in the anterior end of the body as well as the hypodermal sense organs in the same region. This likewise is the destination of at least one pair of nerves leaving each of the ventral ganglia. The branches supplying the hypodermis form a plexus, and thus are united throughout the length of the body, but whether this is also true of those innervating the muscles has not been determined. No nerves have been seen to span the coelomic cavity and thus become attached to any of the contained organs, which therefore may be supplied with branches from the brain and terminal ganglion of the ventral cord.

CLASSIFICATION

Concerning the relationships of the present genus, it has been indicated in the opening paragraphs that it appears to hold a position more or less intermediate between the Echiuroidea and the other classes of annelids, more particularly the polychaetes. Among the annelid features, the nervous system, with its clearly segmented ventral cord, is the most obvious. The head also presents a distinctly annelidan appearance, but until the innervation or the development of this part of the body is known, it is perhaps useless to speculate regarding its homologies. The cirri certainly bear a strong resemblance to those of various polychaetes, while the anterior pair of grooved tentacles is a close counterpart of the bifurcated proboscis of Bonellia with the stalk lacking. Setae are absent, but this is also true of most leeches, and in the Gephyrea armata none have been found in the genera Saccosoma and the more aberrant Epithetosoma. The elements of the body wall are of the annelid and armata type. The extensive coelom, without septa and with a pair of nephridia placing it in communication with the exterior, bears a close resemblance to the perivisceral space of several gephyreans. The coiled gut with gastric or hepatic cells, a ciliated groove in the intestine, anal pouches or glands, and a terminal anus are likewise gephyrean in character.

The circulatory system combines features common to both groups. In the posterior half of the body the dorsal and ventral vessels with their commissures suggest the condition in typical annelids. Anteriorly, there are slight indications of this same feature, but the presence of a dorsal sinus is correlated with extensive modifications, tending in the direction of the gephyreans. In Echiurus there is a dorsal vessel in the anterior end of the body, which posteriorly connects with a peri-intestinal sinus communicating in turn with a ventral vessel. In the present genus, if the dorsal vessel were to disappear posterior to the level of the intestinal sinus and if the gonad were to shift to the ventral vessel, the conditions in the two genera would be essentially the same. Finally, it is significant that in this new species there are eleven body segments, judging by the number of ganglia in the ventral cord. This corresponds exactly to the number of segments in Urechis caupo from the Monterey Bay region, and Rietsch ('86) finds from ten to twelve in forty-eight-day-old embryos of Bonellia minor. The exact number of metameres in Echiurus is open to question. Hatschek ('80) mentions sixteen, including the anal segment, while Baltzer ('17) claims that these are largely if not entirely non-existent in embryos, both from Naples and Messina.

With all of the above facts in mind, the following classification appears to be justified.

Poeobius, new genus. Pelagic organisms, semitransparent, laterally compressed, attaining a length of 27 mm. Head provided with an anterior pair of grooved tentacles, and, more posteriorly, five pairs of cirri. Somatic muscles separated by a fluid-filled cavity from the hypodermis and cuticle. A spacious perivisceral cavity (coelom) without septa, and communicating with the exterior by a pair of anterior nephridia. Mouth subterminal, the buccal cavity communicating with a ventral pouch adjacent to the crop. Gut somewhat coiled, with gastric division and slender intestine leading to the terminal anus. A pair of anal glands present. Circulatory system closed, the dorsal vessel in the anterior half of the body being greatly enlarged and partly filled with four distally branched columns of cells attached to the sinus wall anteriorly. Gonad a development of the coelomic epithelium about the genital vessel in the midsection of the body. Nervous system comprising supra-oesophageal ganglia, connectives, and eleven pairs of ventral ganglia.

Type of the genus, Poeobius meseres.[1]

An extensive search through the literature[2] fails to disclose any closely related genus, nor is there any existing family to which it appears to belong. Accordingly, a new family,

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[1] Ποῖος, of what nature, *βιος*, life; *μεσηρης*, intermediate.

[2] I am under deep obligations to Librarian W. J. Fox for the unrestricted use of the magnificent library of the Philadelphia Academy of Natural Sciences.

Poeobiidae, is herewith established, with the characters of the genus and Poeobius as the genotype. Provisionally, at least, this family is considered to be a member of the class Echiuroidea (Gephyrea armata).

BIBLIOGRAPHY

Baltzer, F. 1917 Monographie der Echiuriden des Golfes von Neapel. Fauna u. Flora d. Golfes v. Neapel, Monograph 34.

Hatschek, B. 1880 Über die Entwicklungsgeschichte von Echiurus und die systematische Stellung der Echiuridae (Gephyrei chaetiferi). Arbeiten Z. Inst. Wien, Bd. 3.

Quatrefages, A. de 1847 Études sur les types inférieurs de l'embranchement des Annelés. Memoire sur l'Echiure de Pallas (Echiurus pallasii). C. R. Acad. Sc. Paris, T. 24.

Rietsch, M. 1886 Étude sur les Géphyriens armées ou Echiuriens. Rec. Z. Suisse, T. 3.

PLATE 1

EXPLANATION OF FIGURES

1 Lateral view of Poeobius meseres, illustrating main features. *ds*, dorsal sinus; *g*, gonad; *h*, hypodermis and cuticle; *k*, nephridium; *sm*, somatic musculature.

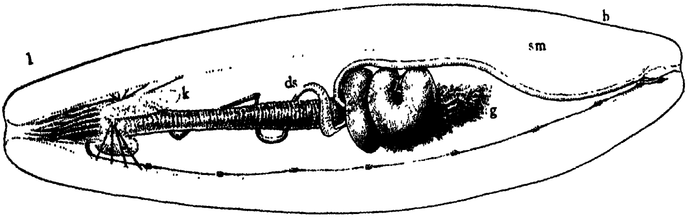
2 Ventral view of head of contracted animal. *ev*, oesophageal vessel; *k*, nephridium; *m*, mouth; *n*, nephrostome; *p*, buccal pouch; *sg*, suboesophageal nerve mass; *v*, ventral cord.

3 Longitudinal section through head region, somewhat diagrammatic. *b*, brain; *c*, crop; *ds*, dorsal sinus; *ep*, elastic pad of buccal pouch, *p*.

4 Lateral view of anterior end of contracted individual. *ds*, dorsal sinus; *ev*, oesophageal vessel; *k*, nephridium; *o*, outer branch of dorsal vessel; *p*, buccal pouch; *pr*, protractors; *re*, retractors; *1*, *2*, *3*, ganglia of the ventral cord.

A PROBLEMATICAL ANNELID
HAROLD HEATH

1



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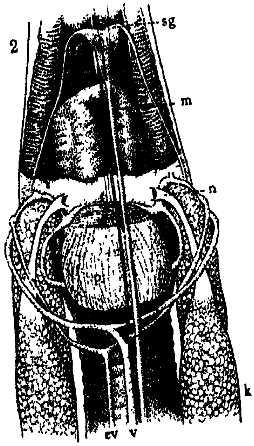

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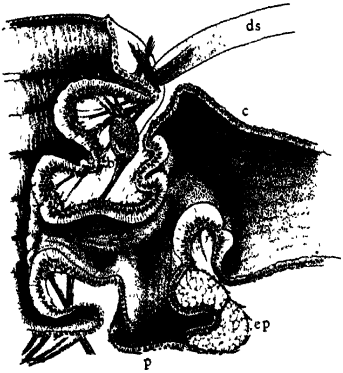

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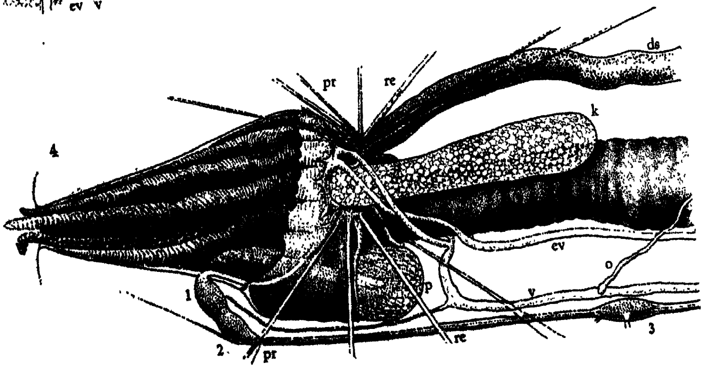


PLATE 2

EXPLANATION OF FIGURES

5 Head of expanded animal, dorsal view; brain shown in dotted outline.

6 Same specimen, ventral view.

7 Same specimen, lateral view.

8 Dorsal view of head region of contracted animal. *ds*, dorsal sinus; *k*, nephridium; *mu*, protractors and retractors.

9 Portion of one of the cords of cells in the lumen of the dorsal sinus; diameter of largest vacuoles, 0.027 mm.

10 Suboesophageal ganglia and next most posterior ganglion.

11 Testis of animal 12 mm. in length; diameter of gonad, 0.19 mm.

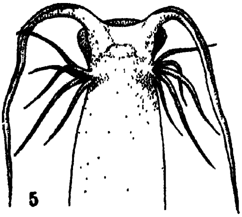
12 Transverse section through snout (*sn*) and tentacles (*t*); *m*, mouth.

13 Anal-gland cells; diameter, 0.04 mm.

14 Section showing genital vessel (*gv*), branching to form a plexus on wall of stomach. *gs*, gastric sinus.

15 Transverse section through (*a*) expanded portion of dorsal sinus and included columns of cells; greatest diameter, 1.26 mm.; *b*, transverse section through dorsal sinus at the point of its anterior bifurcation; diameter, 0.18 mm. The four cell columns, nearly filling the lumen of the sinus, are being proliferated from the sinus walls.

16 Anal gland; *in*, intestine.



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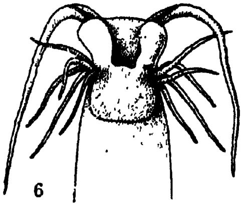

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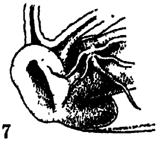

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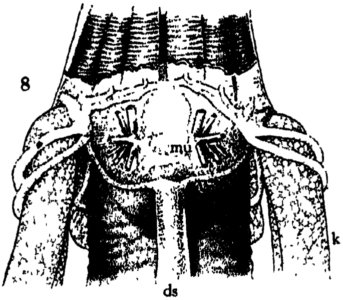

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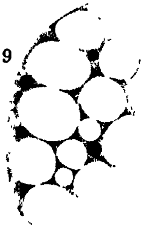

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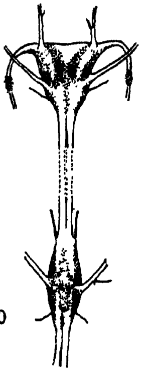

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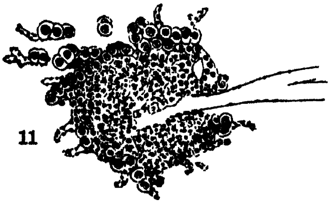

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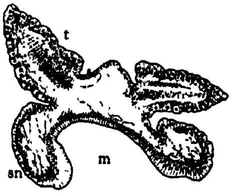

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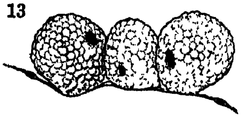

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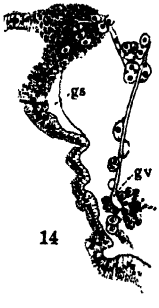

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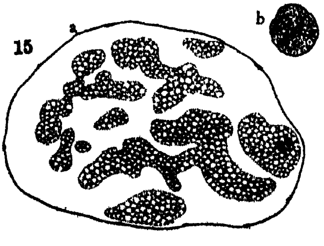

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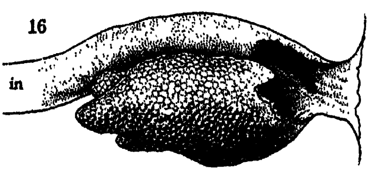

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PLATE 3

EXPLANATION OF FIGURES

17 Section through portion of ovary, showing genital vessel, its branches, and immature and nearly ripe ova with average diameter of 0.1 mm.

18 Section through posterior end of proboscis and anterior end of buccal pouch, *p;* cilia omitted.

19 Hypodermal nuclei, group of nuclei belonging to sense organ, and attached nerve fibers.

20 Transverse section through crop (*c*), and posterior end of buccal pouch with its elastic pad (*ep*).

21 Section through anterior end of a nephridium; *f*, limb connecting with nephrostome.

22 Section through main portion of nephridium.

23 Transverse section through intestine; beneath ciliated groove (cilia omitted from figure) is the subintestinal vessel.

24 Transverse section through a cirrus with two included blood vessels.

25 Transverse section through (*a*) a ventral ganglion and (*b*) a pair of ventral connectives.

26 Portion of body wall, showing an outlying membrane (*bm*) of unknown function, the coelomic epithelium (*ce*), circular (*cm*) and longitudinal (*lm*) somatic muscles.

27 Gastric epithelial cells, 0.08 mm. high.

28 Section through gonad of animal 5 mm. in length; greatest diameter of gonad, 0.06 mm.

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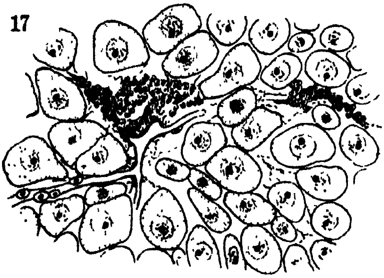

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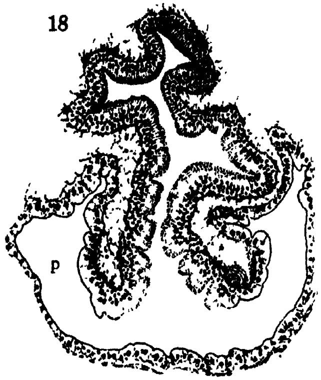

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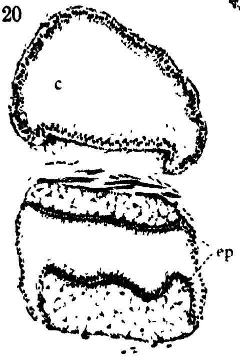

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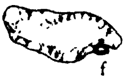

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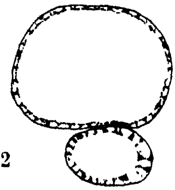

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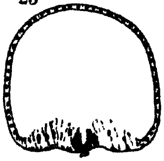

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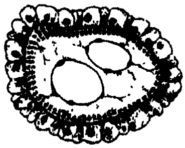

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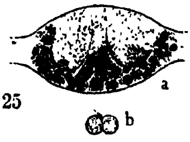

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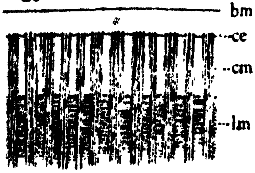

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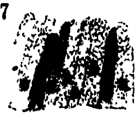

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STUDIES ON SOME PHYSIOLOGICAL FUNCTIONS AND MORPHOLOGICAL CHARACTERS OF RHABDITIS (RHABDITIDAE, NEMATODES)

B. G. CHITWOOD
George Washington University

EIGHT TEXT FIGURES AND THREE PLATES (TWENTY-FOUR FIGURES)

AUTHOR'S ABSTRACT

Intravitam stains were used to determine the functions of several organs in two species of nemas (Rhabditis strongyloides and Rhabditis elongata). The organs were also studied in section. From the results obtained it is concluded that the amphids are not excretory in function, but more probably sensory, for definite connections were observed to extend to the nerve ring. No migratory cells, such as those described by Stefanski, were seen.

The phasmids stained with all intravitam stains used, but were never observed to secrete. It seems doubtful that they serve as excretory organs

The excretory system was seen to consist of a typical X system. Actual excretion was observed. Deirids were seen for the first time in both species. Oesophageal glands were also described. A study was made of the structure of the intestinal cells, rectal glands, and anal muscles. Attention was called to the fact that there are two kinds of ejaculatory glands, one of which probably serves as a 'cement gland,' while the function of the other is still in doubt.

INTRODUCTION

This work was done under the direction, and with the criticism, of Dr. Paul Bartsch, and to him I express my appreciation. I wish also to thank Dr. N. A. Cobb and Dr. G. Steiner for valuable suggestions and criticisms.

It was begun with the intention of studying only the amphids and phasmids, but in so doing my attention has been drawn to several other organs.

The amphids are the same structures which have been listed under the name 'lateral organs' by earlier writers. In the parasitic forms they are usually quite small, and were overlooked in numerous instances. Goldschmidt(7) figures such organs in Ascaris megalocephala and A. lumbricoides showing definite innervation, but regards them as specialized papillae. Cobb has seen them in most of the parasitic and free-living forms which he has examined, while Steiner figures amphids with sensory terminals in Tylenchus, Aphelenchus,

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Cephalobus, and other genera of plant parasitic and free-living forms. Thus it may be said with a reasonable degree of surety that amphids are universal among nematodes.

The external features of the amphids vary greatly, the largest being seen among the mermithids and also some marine genera. When they are helicoid the right amphid always turns left and the left one, right. Their shape is often diagnostic. Cobb has found that the spiral is probably the most widespread form of amphid and in the marine nemas the so-called 'circular' amphids are usually overlapped spirals. For convenience he classes amphids under the following heads: spiral, stirrup-shaped, and 'circular.' Filipjev goes even so far as to make the amphids one of the characters upon which to base a general classification of nemas.

In Rhabditis, Tylenchus, Aphelenchus, Cephalobus, and other genera the external amphids are minute and may easily be overlooked. They appear as minute pores or papillae. Cobb suggests the possibility that the amphids and phasmids are derived from a series of lateral organs, of which the amphids and phasmids are the only ones remaining. He cites Agamermis decaudata as an example in which the first two lateral organs are well developed, while there are several progressively less developed but morphologically similar organs posterior to the first pair.

He believes, furthermore, that the lateral ducts of the excretory system are developments from the ventral gland which have come to lie in the lateral chords, because in that situation they are subject to the least movement.

The function of the amphids has never been settled. Some believe them to be organs of balance, others, of respiration, while still others maintain that they are chemical sense organs. Stefanski considers them excretory organs. The present conception is that they are sensory organs of some type, probably chemotactic.

This latter view was probably first put forth by zur Strassen(21), who, under the name of 'Seitenorgane,' described and figured the amphids of Anthraconema. He drew the innervation in detail. Later, Steiner described the sensory terminals of a large number of nemas in "Nematoden der Tiefenfauna des Neuenbürgersees." In 1924, Cobb described the amphids of Caconema radicicola, attributing to them the function of chemical sense organs, while, in 1925, Steiner(19) went further into the subject with Heterodera schactii and Tylenchus dipsaci. In the latter work he calls attention to the remarkable ability of the nema to locate its host plant, which could only be explained by action of such organs. Glands have been associated with the amphids in many cases. These glands are believed by some to act in a manner similar to glands of chemical sense organs in other animals as a means of irrigating the sensory surface. I have at times thought that I recognized a small gland connected with the sheath of the amphidial nerve at the level of the first bulb, but this was quite indistinct, and in sections there was nothing conclusive.

Stefanski(16) has figured amphids in Rhabditis stained with various intravitam stains. He believes that certain cells of the second bulb of the oesophagus may migrate into the body cavity, and up to the amphids, where they emerge, carrying with them excretory products.

The phasmids, like the amphids, lie in the region of the lateral chords and open to the exterior by means of a short duct. They were first defined by Cobb as a pair of obscure organs, hence phasmid-ghost thing, located posteriorly, nearly always behind the anus near or in the lateral line.

Stefanski attributes an excretory function to them also, while Cobb now gives them a glandular function. They are described in numerous species, but often they are not seen in the male, especially in those forms which have a complete bursa surrounding the tail.

Steiner(18) believes the amphids and phasmids to be derived from the retrocerebral apparatus and postlateral antennae, respectively, of the Rotifer. Such paired organs as the amphids with nerve endings are rare and the similarity is remarkable, as is also the similarity of the excretory systems of the two types. Maupas(9) described what he believed to be flame cells at the end of a longitudinal duct in a Rhabditis icosiensis female. If they are present they would give additional information on the phylogeny of nemas. The lateral excretory canals, according to Steiner(18), show various gradations, indicating changes from a paired system opening into the cloaca. He cites the H or X system of Rhabditis as one of the more primitive forms. The unicellular type of excretory system seen in some marine forms was derived from this by gradual degeneration of the lateral tubes.

MATERIAL AND METHODS

In order to determine to what extent these organs enter into the excretory activities of the nemas, I have studied two species of Rhabditis, namely, Rhabditis strongyloides and Rhabditis elongata. Both species were cultivated on nutrient agar and on beef-infusion agar. For use in sectioning for some of the smaller organs I found that the nemas could be grown larger by keeping them on a medium composed of 1.5 grams agar to 100 cc. of thick broth from boiled beef.

For sectioning I have fixed the nemas in formalin, Flemming's strong, osmic acid, and a solution of osmic acid one part and saturated solution of bichloride of mercury one part. For stains, Delafield's haematoxylin and eosin, Heidenhain's haematoxylin, Apáthy's method, and Bethe's methylene-blue technic were used.

Inasmuch as I have used Rhabditis elongata Schn. in some of these experiments, while Stefanski stained Rhabditis tenuicaudata, I would like to call attention to the probable synonomy of these species. Two characters of the male form the basis for distinction. These are characters which might be easily mistaken or overlooked. The first, in Rhabditis elongata there are six postanal papillae, while in Rhabditis tenuicaudata only five are described. The first postanal papillae are difficult to see. Other papillae are identical. The second, in Rhabditis elongata the spicule is split for two-thirds of its length, while that of Rhabditis tenuicaudata is figured to be intact. However, unless one mashes the spicules out of the body under a cover-slip, one might never notice that the spicules of Rhabditis elongata are split. There is quite a wide variation in size of the body of Rhabditis elongata. This depends largely on the amount of nutritive material in the media and the number of nemas living on it. I will give the formulae of both Stefanski and Reiter. My own vary within the ranges of both.

Menzel and Stefanski's formula for Rhabditis tenuicaudata

L 1.378 mm.	α 27	β 6	γ 5
L 2.206 mm.	α 28	β 6	γ 6

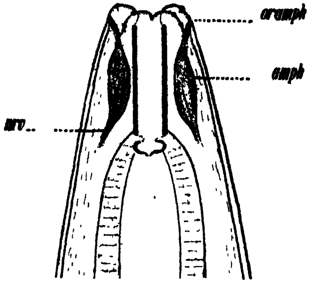
Reiter's formula for Rhabditis elongata

L 1.5(0.8–2.9)mm.	α 23.3(21–32)	β 7.2(5–9)	γ 4.5(3.2–5.6)
L 1 (0.7–1.5)	α 26 (24–28)	β 5.5(4.5–6.5)	γ 5.6(4–6.7)

THE AMPHIDS

In carmin indigo I have watched the stain as it penetrates the two species, and can state that the amphids and phasmids are both stained from the outside. The stain shows first on the edges of the duct near the opening. It gradually stains the neighboring tissue. The stain does not show in the body cavity until after the amphids are distinctly colored. There is a distinct continuation down the lateral line. Only the beginning of this may be seen with carmin indigo. Cells of the lateral line may take up the stain, forming vacuoles. This occurs only after prolonged staining and may indicate degeneration.

Neutral red was quite useful, because it stained the amphids quickly. In those cases in which it is practical this dye will differentiate the amphids from the papillae. Papillae are stained at their termination and for a short distance internally as a fine red line, while the amphids are 'filled' with the stain. It stains granular regions in the oesophagus very heavily. The most granular staining bodies are probably nuclei, while some masses of stain show brownian movement as in vacuoles. I have watched the manner in which the stain goes through the oesophageal wall into the body cavity, and have never seen anything which appeared to be a migratory cell, as Stefanski(16) describes. The stain diffuses through the oesophageal walls and stains the granular masses most heavily.

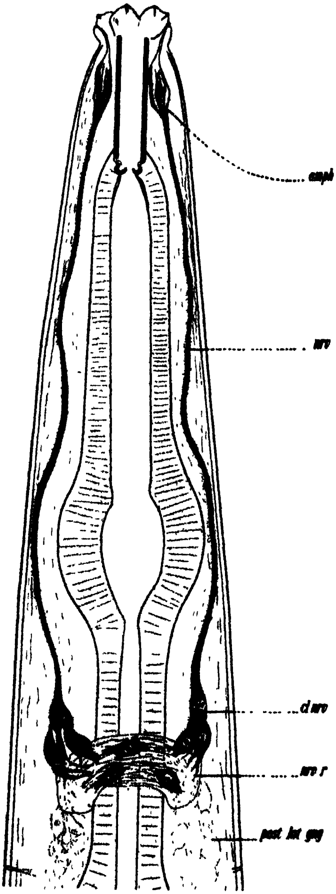


Text fig. A Head of Rhabditis elongata stained with carmin indigo. × 900.

ABBREVIATIONS FOR ALL FIGURES

ac, accessory piece
amph, amphid
ant lat gng, anterior lateral ganglion
ant ren, anterior lobe of renette cell
brs, bursal muscle
cap sp, head of spicule
clc, cloaca
chrd dsl, dorsal chord
chrd lat, lateral chord
chrd vnt, ventral chord
cl nrv, nerve cell of amphid
crd, cardia
dct ej, ejaculatory duct
dct ex, excretory duct
dst gl mn, duct of small ejaculatory gland
dct lat, lateral excretory duct
dct ph, tube of the phasmid
dct ren, duct of the renette cell
dr, droplet of excreted material
dsl msc an, dorsal rectal muscle
dsl msc rct, dorsal rectal muscle
frb, nerve fiber
glob, globule of stored food
gl oe, oesophageal gland
gl dsl an, dorsal rectal gland
gl subv an, subventral rectal gland
grn, stained granule
int, intestine
lum, lumen of renette cell
msc ncl, nucleus of muscle cell
msj ej, ejaculatory muscle
msc vnt an, ventral rectal muscle
msc oe, muscular part of oesophagus
ncl, nucleus
ncl ren, nucleus of renette cell
nrv, nerve
nrv r, nerve ring
oe, oesophagus
or amph, orifice of amphid
or ph, orifice of phasmid
p ex, excretory pore
ph, phasmid
ph cl, cell of phasmid
post lat gng, posterior lateral ganglion
post ren, posterior lobe of renette cell
ppl, papillae of head
rct, rectum
ren, renette cell
sp, spicule
sp ext, extensor spiculorum
sp ret, retractor spiculorum
tb amph, tube of amphid
t ex, excretory duct
trm, terminal fibers
vac, vacuole

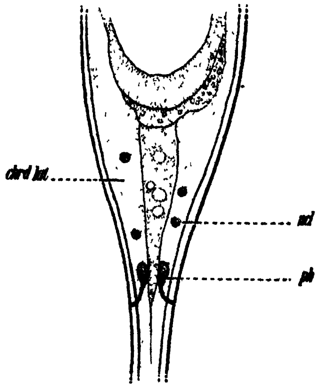
Methylene blue was useful in staining both the amphids and their nerves. In the living specimen distinct terminals or sensory fibers could be seen in the amphids. Five or six could be distinguished in each. These have a slight thickening in the center, posterior to which they come together in the amphidial nerve (text fig. B), and are surrounded by a sheath. The amphidial nerve stains a deep blue, while the



Text fig. B Anterior end of Rhabditis strongyloides stained with methylene blue. × 715.

surrounding tissue is somewhat lighter in color. No separate nerve fibers can be distinguished in the nerves, but in Rhabditis strongyloides each nerve breaks up into six fibers which pass to six corresponding cells anterior to the nerve ring. These cells have axons leading into the nerve ring.

The nerve ring did not stain, and the posterior ganglion stained only rarely. The cells associated with the amphidial nerve stained more readily in Rhabditis strongyloides than in Rhabditis elongata. I have corroborated the intravitam methylene-blue stains with horizontal, longitudinal, and with cross-sections.

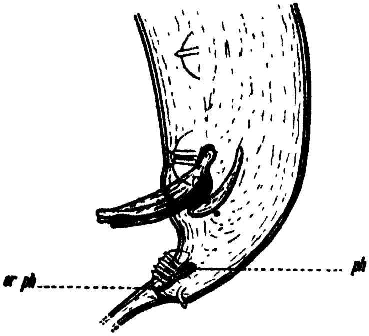


Text fig. C Tail of Rhabditis elongata female, dorsal view, stained with carmin indigo. Nuclei in the lateral chords are stained, but they seem to have no functional connection with the phasmids. × 600.

Ammoniacal carmin and carmin rubrum were also used, both staining the amphids with results similar to those obtained with carmin indigo.

In section the two species are so similar in the anterior region that there is no advantage in figuring both. Rhabditis strongyloides is a little larger, and for that reason shows some structures better. There are six lips in this species, each of the dorsal and ventral lips having two papillae, one on the summit and one on the side. The lateral lips have one papilla each, the outer one being replaced by the amphids.

The external amphids are minute pores (fig. 1). Small tubes lead into the amphidial pouch or sac. Arranged in the thick wall, one may see what appears to be six terminals (fig. 3). Upon leaving the amphids, these are at first in two groups which unite, forming the amphidial nerve which may be seen enclosed in a sheath. This runs through the dorsal part of the lateral line. The nerve breaks up into six parts which lead to a like number of cells. Probably these are the cell bodies, the terminals of which may be seen in the amphidial pouch. They are in direct relation with the nerve ring (figs. 4 to 6).



Text fig. D Side view of male Rhabditis elongata, showing the phasmids stained with neutral red. × 600.

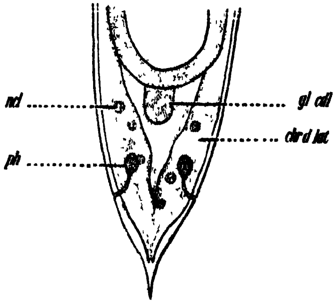
THE PHASMIDS

The phasmids of Rhabditis elongata males and females consist merely of a tube leading to the inner side of the lateral chord where one finds a cell embedded. They appear to be much as Stefanski describes them, except that it seems very doubtful that the cells seen stained with carmin indigo in the lateral chords just anterior to the phasmids take any part in their activity. I have never seen the phasmids secrete; if they do so, it is not as continuous as the secretion emptied through the excretory pore.

Carmin indigo and neutral red stain them quite well. The body of the organ appears deeply granular.

The phasmids in Rhabditis strongyloides are so similar in structure and activity that the same description may hold for them (text fig. E).

Methylene blue acts in a manner similar to neutral red and carmin indigo on the phasmids, but on several occasions I have noticed what appeared to be a bipolar neurone innervating a small bristle on the inside of the tube of the phasmid near its opening. This cell connects anteriorly with a ganglion just anterior to the anus. As I have not been able to see it consistently, I have not figured it.



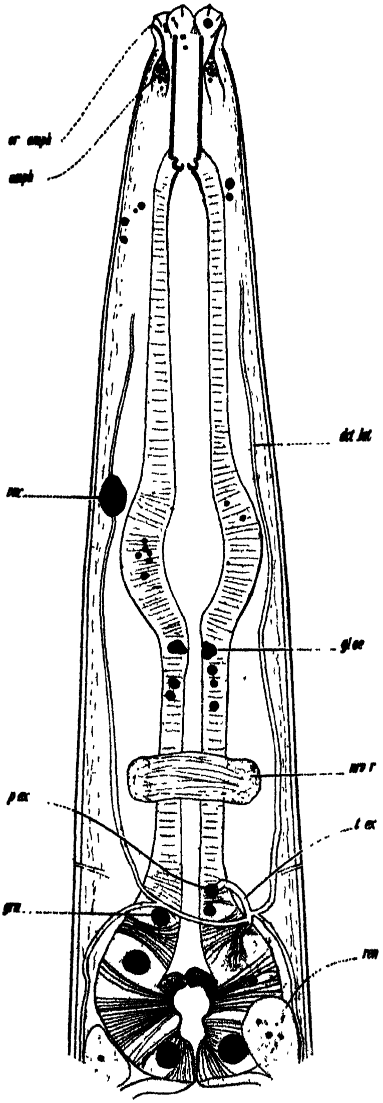
Text fig. E Dorsal view of female Rhabditis strongyloides stained with carmin indigo. The phasmids, their tubes, and some regions in the lateral chords absorbed the stain. The dorsal rectal gland may also be seen, but it is unstained. × 600.

The phasmids may be seen a few sections posterior to the anus. In this region the lateral chords are enlarged as is also the ventral, but the dorsal chord is quite small. The cells connected with the phasmids are embedded in the lateral lines (fig. 22). The ducts of these cells bend inward (fig. 23), after which they make a decided turn and pass to the outside (fig. 24).

THE EXCRETORY SYSTEM

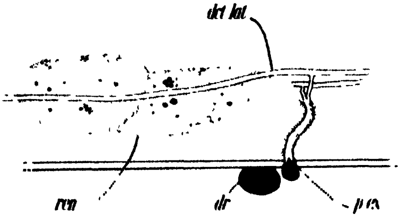
In Rhabditis strongyloides the excretory system consists of two longitudinal ducts running along the ventral side of the lateral chords, two renette cells, and an excretory duct. The excretory system of Rhabditis elongata is more difficult to see than that of Rhabditis strongyloides, but in the former species lateral ducts, paired renette cells, and an excretory pore were identified. The sides of the excretory duct of Rhabditis strongyloides became a light red when stained with neutral red. The two renette cells (ventral glands), one on each side, ventral to the cardiac bulb often form vacuoles. The branches running into the two renette cells were stained also. The lateral excretory tubes at times took a deep red color. This is contrary to the results obtained by Mueller in Ascaris megalocephala and lumbricoides. This author states quite definitely that in injected specimens the dye did not stain the lateral excretory tubes. He, moreover, does not believe that the organs grouped together and now called the excretory system of Ascaris in text-books have an excretory function. Though large amounts of fluids are secreted at the excretory pore, his experiments have brought him to the conclusion that they are not acid, as might be expected if the waste products of nemas are of a similar composition to those of man. But even so, in man the urine in not constant in reaction and may depend somewhat on his health, diet, and general metabolism. Likewise, he found that the ventral gland and duct of Aniskus simplex did not take part in the excretory activities, and as the excretory pore is found quite anteriorly, he suggests that its function may be salivary.

Concerning the function of the aforementioned group of organs, the weight of evidence has in the past, in a general way, pointed toward an excretory function. Cobb has furnished some of the strongest proof. He chemically identified some uric-acid crystals obtained from the lateral chords of Ascaris, while he found proportionally a much lower amount of uric acid in the surrounding tissues. He explains the presence of small amounts of uric acid elsewhere as due to the diffusion of that substance after the death of the nema. In Rhabditis strongyloides stained with neutral red the excretion appears first as a minute droplet at the opening of the excretory pore. This droplet swells to a considerable size (text fig. G). It is a very dark red and must to some extent be insol-



Text fig. F Ventral view of the oesophageal region of Rhabditis strongyloides stained with neutral red. The excretory system and certain regions of the oesophagus take up the stain. × 715.

uble in water, for it does not immediately go into solution, but may slide down the surface of the cuticle. Another drop, forming later, may follow and the two fuse before the droplet disintegrates. These droplets do not show any granular structure, but are homogeneous, very deep red, and liquid. After having seen them in the stained condition, it was rather easy to identify them unstained, in which condition they are clear and transparent. Because of the property of neutral red to become deep red in the presence of acid, it is probable that the secretion is of an acid nature. Further experimentation along the same line makes me more confident of the reaction of the secretion. Specimens which were placed in neutral red and mounted immediately showed a deep red droplet at the excretory pore. The stain did not penetrate the inside of the nema, and for that reason the fluid must have been stained from the outside. This result would be logical only if the droplet were acid. Likewise, I have seen a deep red droplet at the excretory pore of Rhabditis elongata.



Text fig. G Lateral view of the excretory organs of Rhabditis strongyloides, showing secretion in the living specimen. × 900.

The renette cells may become a deep blue after the nema has lived in the methylene blue for several days.

In sections anterior to the cardiac bulb one may note a pair of small tubes in the ventral part of the lateral chord. These are the longitudinal excretory tubes, and may be seen to connect with the common excretory duct near the level of the excretory pore. They are quite distinct in this region, but become so small anteriorly and posteriorly that they could no longer be distinguished, though in living specimens they were traced anteriorly to a point not far from the base of the pharynx and posteriorly to two or three cells just anterior to the anus. The common excretory duct leads inward, where it is inclosed in the ventral chord which unites at this place with the two lateral chords, forming a bridge. It extends into one of the lateral chords, forming a short loop, and returns, dividing into two tubes, which unite with the lateral excretory tubes, and into two short ducts going into the renette cells which stain heavily and may overstain with haematoxylin. They are reflexed upon themselves, for which reason they often seem to be four cells instead of two in the living animal. The posterior continuation of the longitudinal tubes lies in close relation to the renette cells. The anterior lobe of the renette cell contains the nucleus and is traversed by a duct. This duct continues into the anterior part of the posterior lobe of the renette cell. The size and exact shape of these cells seem to vary quite markedly, though they nearly always extend from the beginning of the second bulb to the middle of the cardia, lying symmetrically on the two sides of the ventral chord (figs. 7 to 10).

THE DEIRIDS

In Rhabditis strongyloides small organs may be seen at a latitude of 15, which cause a distinct break in the cuticle similar to the opening of a duct. They pass into the lateral chord and posteriorly for a short distance to the ganglion just anterior to the beginning of the cardiac bulb. It is possible, though difficult, to see these organs in the living specimen; however, they are quite obvious in a horizontal longitudinal section. Deirids were also seen in Rhabditis elongata, in which they are just posterior to the nerve ring. These organs did not stain in either species with any of the intravitam stains used, nor was a nerve fiber distinguished, but in all probability such was present.

THE OESOPHAGEAL GLANDS

Regions in the oesophagus which are of a non-muscular nature take up stain heavily when neutral red or carmin indigo is used. Some of these non-muscular regions may contain the nuclei, which would explain their affinity for certain stains, while other regions may contain oesophageal glands. Three of these were seen at the base of the first bulb in both Rhabditis strongyloides and Rhabditis elongata. One is dorsal and the other two are subventral. They are located within the musculature of the oesophagus. Each is bilobed, and a definite duct was seen entering the lumen from each gland.

THE INTESTINE

In section of the cardia of Rhabditis strongyloides one sees that four thin cells make up the circumference. The nuclei are elongate ovals. On the inner side of the cells there is a low border of more or less fused rod-shaped structures called by some authors the 'Stäbchensaum.' There are no stored food globules in this part. Immediately behind the cardia the cells are much thicker, and six or eight are required to make up the circumference. The rotund cells protrude into the lumen, their inner border showing the small rods quite plainly; often the rods show individually, being separated at their tips. Throughout the intestine the nuclei are central and slightly ovoid, those of all the cells show in the same section usually. Posteriorly, four cells, not quite as heavily built, and with longer rods simulating a striated membrane, may be seen. Stored food globules seem to make their first appearance in this region. This state of the intestine continues for three cell lengths, and then the wall is made up of just two cells. It is here that the globules are most numerous, and during life it appears dark brown. The prerectum also has a wall composed of four cells, containing little or no food, like the cardia (fig. 12).

Fixation with osmic acid and counterstaining with eosin without bleaching show the structure of the lining very well.

The rods are often distinct, and at their base there is a row of closely packed granules, inside of which there is a homogeneous zone. The food globules do not appear by this method of preparation thus, the cell substance seems quite alveolar (fig. 14).

THE RECTAL GLANDS

At the posterior end of the intestine there are three large unicellular glands, one the dorsal, the other two the subventral rectal glands. Of these the dorsal is slightly the largest. They empty into the rectum a short distance anterior to the anus. The dorsal one may lie with its distal end slightly posterior to its opening or it may lie as the others with its distal end directed anteriorly. The function of these glands is not known. They do not stain with the intravitam stains which were used. It is interesting to note that the same symmetry exists here that is present in the oesophageal region (figs. 16 to 21).

THE MUSCLES IN THE ANAL REGION

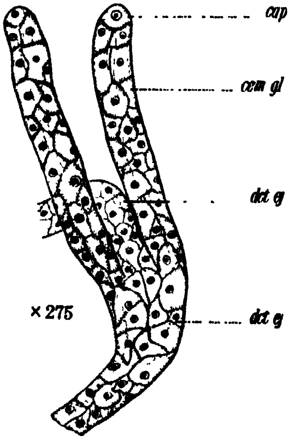
In the female two large dorsal muscles extend from the dorsolateral wall to the rectum. Several smaller muscles extend from the ventrolateral region.

In the male the scene is somewhat more complicated by the musculature of the spicules. Each spicule is embedded in a mass of material—its pouch. The spicules unite posteriorly, forming a trough, and have but one point. The gubernaculum appears as a slight semicircle in section. Dorsolateral muscles, retractores spiculorum, extend from the heads of the spicula. Muscles from each ventrolateral region, extensores spiculorum, are also present. Another set of muscles, probably the bursal muscles, run obliquely from the ventrolateral region to the ventrosubmedian muscle region (figs. 19 to 21).

EJACULATORY GLANDS

Schneider first described two large glands emptying into the ejaculatory duct in a Rhabditis strongyloides male. Cobb has since(5) described glands which are probably similar in Rhabditis icosiensis, both authors attributing to them the function of secreting cement. Steiner has described 'cement glands' in several forms. Their cellular structure was, however, not discernible. I have previously(1) given a histological description of an ejaculatory gland in Rhabditis strongyloides under the name 'cement gland.'

Since that time I have been able to distinguish another pair of glands, much smaller than the first and likewise opening into the ejaculatory duct, but somewhat more posteriorly. The difference in the structure of the two organs makes it doubtful that they have the same function; therefore, it is proposed that, until distinct evidence as to which may be the cement gland is forthcoming, they be known as large ejaculatory glands and small ejaculatory glands, the first name to be applied to the large multicellular gland described by Schneider, opening at some distance anterior to the cloaca, while the latter term is applied to the smaller two-celled organs emptying farther posteriorly.



Text fig. H Large ejaculatory glands of Rhabditis strongyloides. × 275.

LARGE EJACULATORY GLANDS

Two large cylindrical glands opening into the ejaculatory duct approximately two and one-half anal body diameters anterior to the anus and extending 15 per cent the length of the body (135 μ). In the living specimen they are transparent, a light gray in color. The lumen is surrounded by six cells for the greater part of its distance, but at the extremity two cells make up its circumference, while a single cap cell terminates the organ. The structure of the cells is very close to that of the cells making up the ejaculatory duct.

SMALL EJACULATORY GLANDS

Two glands emptying into the ejaculatory duct just anterior to or even with the rectal glands. Each gland is composed of two cells which make up the body of the gland. An intracellular lumen extends posteriorly from the middle of the first cell. The body of the cell is approximately one and one-half anal body diameters in length. In the living specimen it is practically indistinguishable, but a longitudinal section stained with haematoxylin shows them quite well.

BIBLIOGRAPHY

1 Chitwood, B. G. 1929 Notes on the copulatory sac of Rhabditis strongyloides Schn. Helm. Soc. of Wash., 117, Jour. Parist., vol. 15, no. 4.

2 Cobb, N. A. 1898 Extract from a manuscript on the nematodes parasitic on live stock. Agric. Gazette of New South Wales.

3 ———— 1914 Nematodes and their relationships. Yearbook of Agric.

4 ———— 1917 Notes on Nemas. Contributions to a science of nematology. V. Waverly Press.

5 ———— 1925 Note on Rhabditis icosiensis. Helm. Soc. of Wash., 78th meeting. Jour. Parist., vol. 11, pp. 217–230.

6 Filipjev, I. 1929 Proceedings of the Helm. Soc. of Wash. (D. C.). (Secretary's abstract.)

7 Goldschmidt, R. 1908 Das Nervensystem von Ascaris lumbricoides und megalocephala. Zeit. für wissenschaftliche Zoologie, Bd. 90.

8 Hetherington, D. C. 1923 Comparative studies on certain features of nematodes and their significance. III. Biol. Mono., vol. 8, no. 2.

9 Maupas, Nouveau 1916 Rhabditis d'Algerie. C. R. Soc. Biologie, T. 79, p. 607.

10 Menzel et Stefanski 1917 Contribution à l'étude de l'excrétion chez les Nematodes libres. Biologisches Zentralblatt, 37. Bd., Nr. 6.

11 MUELLER, JUSTUS F. 1929 Studies on the microscopical anatomy and physiology of Ascaris lumbricoides and Ascaris megalocephala. Zeit. für Zellforschung und mikroskopische Anatomie, Abt. 8 der Zeit. für wissenschaftliche Biologie 8. Bd., 3. Heft.

12 RAUTHER, MAX 1907 Über den Bau des Oesophagus und die Lokalisation der Nierenfunktion bei freilebenden Nematoden. Zool. Jahr., Bd. 23, H. 4.

13 REITER, MATTH. 1928 Zur Systematik und Oekologie der zweigeschlechtlichen Rhabditiden. Zool. Institut d. Universität Innsbruck, Bd. 3, H. 4.

14 SEURAT, L. G. 1920 Histoire naturelle des Nematodes de la Berberie, première partie. Zool. générale, Université d'Alger.

15 STEFANSKI, W. 1922 Excretion chez les Nematodes libres. Disciplinarium Biologicarium Archivum Societatis Scientiarum Varsaviensis, T. 1, Fasc. 6.

16 STEINER, G. 1919 Untersuchungen über den allgemeinen Bauplan des Nematodenkörpers. Zool. Jahrb., Anat., Bd. 43, S. 1–96.

17 ———— 1919 Nematoden der Tiefenfauna des Neuenbürgersees. Bull. de la Société des Sciences Naturelles, T. 43.

18 ————1920 Betrachtungen zur Frage des Verwandtschafts-Verhaltnisses der Rotatorien und Nematoden. Festschrift für Zschokke, Bd. 31.

19 ———— 1925 The problem of host selection and host specialization of certain plant-infesting nemas and its application in the study of nemic pests. Phytopathology, vol. 15, no. 9.

20 ZUR STRASSEN, O. L. 1904 Anthraconema, eine neue Gattung freilebender Nematoden. Zool. Jahrb., Sup. 7, S. 301–346.

PLATE 1

EXPLANATION OF FIGURES

Cross-sections of Rhabditis strongyloides stained with haematoxylin and eosin. Figures 1 to 4, × 1240; figures 5 to 8, × 975.

1 Lip region. The papillae and amphidial orifice may be seen.

2 Base of the lips. The amphidial tube runs through the two lateral lips.

3 Section through the amphid proper or internal amphid. The amphidial tube is surrounded by a condensed mass of tissue in which the nerve terminals are embedded.

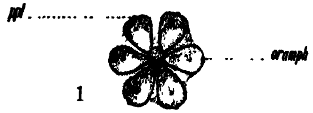
4 Anterior oesophageal region. The nerves have not yet fused into a single nerve. They lie in a condensed sheath-like mass.

5 Section taken not far anterior to the nerve ring. Some of the amphidial nerve cells and some nerve fibers may be seen. The lateral excretory ducts are also present.

6 This section is through the anterior or dorsal part of the nerve ring. Some of the fibers from the amphidial nerve are about to enter it.

7 Section through the excretory pore, showing duct and lateral excretory tubes.

8 Taken slightly posterior to the preceding section. This shows the beginning of the paired renette cells and the posterior wall of the excretory duct, due to a slight bend in the latter.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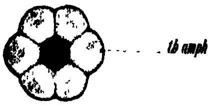
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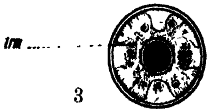
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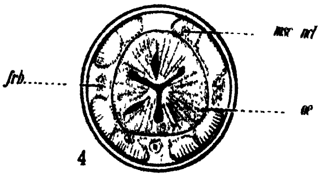
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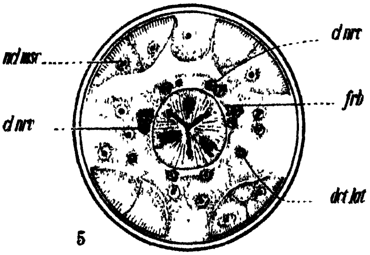
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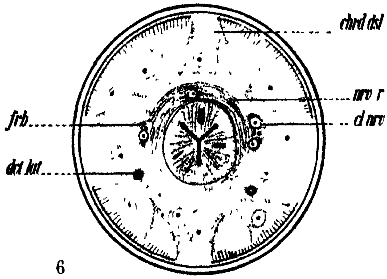
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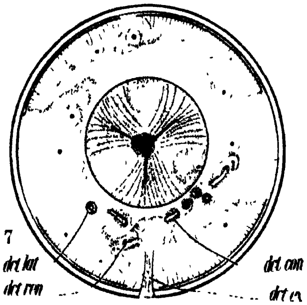
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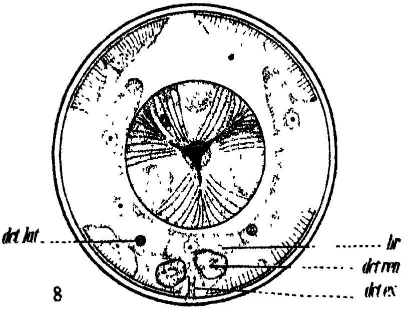
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PLATE 2

EXPLANATION OF FIGURES

Rhabditis strongyloides

9 The anterior lobe of each renette cell with its nucleus is conspicuous at the level of the cardiac bulb. Each cell has an indistinct lumen. × 975.

10 Each renette cell is reflexed. The longitudinal excretory tubes are situated in the lateral lines, but in very close approximation to the renette cells. Farther posterior, they assume a position more nearly in the center of the lateral lines. The lumen persists into the posterior lobes of the renette cells, but become less distinct. × 975.

11 Cross-section of Rhabiditis strongyloides male, showing glands ejaculatory major. × 800.

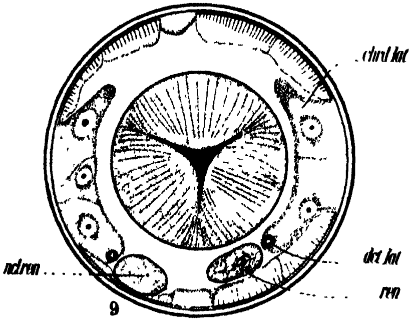
12 The intestine of Rhabditis strongyloides. Rods may be seen bordering the lumen. Beneath these there is a row of granules. × 975.

13 Glands ejaculatory minor as seen in a longitudinal vertical section of Rhabditis strongyloides male. × 975.

14 Intestinal cell of Rhabditis strongyloides as seen in a longitudinal section stained with osmic and eosin. × 975.

15 Tail of Rhabditis strongyloides female, showing rectal glands. × 875.

PLATE 2



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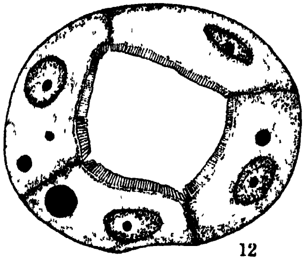

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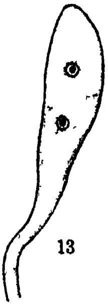

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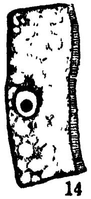

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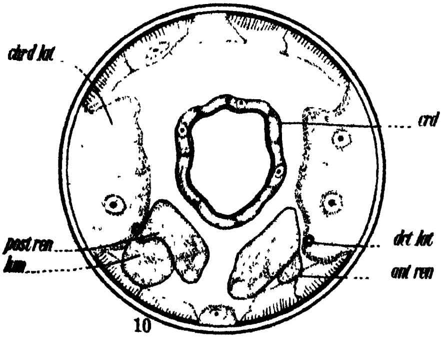

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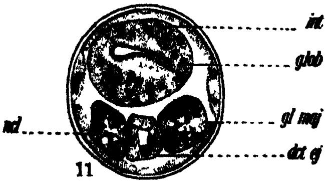

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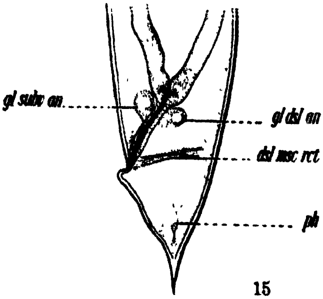

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PLATE 3

EXPLANATION OF FIGURES

Tails of Rhabditis strongyloides males and females stained with haematoxylin and eosin.

16 Female, showing opening of one gland into the rectum. × 975.

17 Female, farther posterior, showing dorsal rectal gland. × 975.

18 Female, still farther posterior, showing dorsal rectal gland. × 975.

19 Male, through head and spicula. × 975.

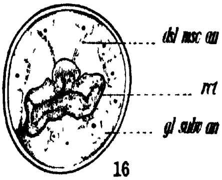
20 Male. Two sections posterior in same series. × 975.

21 Male. Two sections farther posterior in the same series. × 975.

22 Female. The phasmid cell may be seen. × 1200.

23 Section just posterior to figure 22. × 1200.

24 Next section posterior to figure 23. The left tube opens to the outside, while the right one does so at a slightly different level, as the section was more or less oblique. × 1200.



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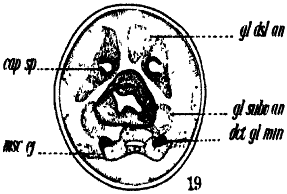

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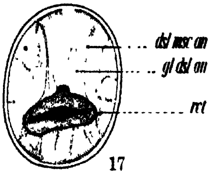

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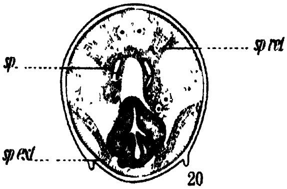

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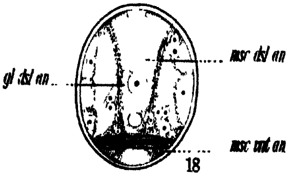

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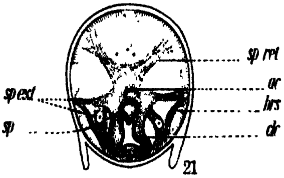

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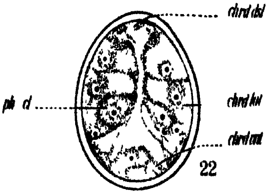

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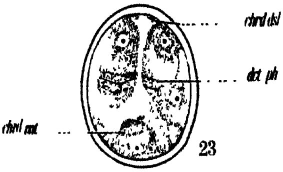

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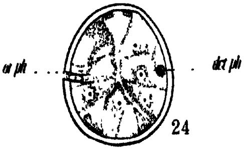

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THE FORMATION OF THE SEX GLANDS AND GERM CELLS OF MAMMALS

III. THE HISTORY OF THE FEMALE GERM CELLS IN THE ALBINO RAT TO THE TIME OF SEXUAL MATURITY

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FIVE PLATES (THIRTY-NINE FIGURES)

AUTHOR'S ABSTRACT

The entire ovary, including its germ cells, is produced by a local proliferation of peritoneal cells. The germ cells of the embryonic ovary grow, divide, become grouped into nests, pass through synaptic changes, and become primary follicles by three days after birth. At that time, growth of all the ova and follicles begins, and this results in a normal maturation, then a degeneration of all the ova by about thirty-two days after birth; few, if any, of the original germ cells remain after this degeneration. About twenty-three days after birth, there begins a great activity of the germinal epithelium in forming new ova, reaching its maximum between thirty-six and thirty-nine days, but continuing into the adult animal.

The definitive ova of the adult are transformed peritoneal (germinal epithelial) cells formed anew during the late youthful and adult life. This occurs chiefly by a local enlargement of single germinal epithelial cells which become surrounded by follicle cells and push into the ovary; there is also the production of ova from ingrowing cords of the surface layer of the ovary. The original germ cells pass through synapsis and other meiotic changes in late embryonic and early postnatal periods, but these all degenerate; synapsis cannot be distinguished later than three days after birth. But it is possible to follow, in young and adult ovaries, the transforming germinal epithelial cells into ova which pass through normal maturation and ovulation; therefore, these must be considered as true ova, which they are in fact, even though synapsis cannot be observed in their history.

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INTRODUCTION

In an earlier study (Hargitt, '25) covering the origin of the germ cells, it was found that the gonad is formed by a proliferation of the coelomic or peritoneal epithelium at about the time the midgut closes to form a tube, producing a thickened genital ridge with intermingled large and small cells. All the cells of the genital ridge have the same origin from the ordinary coelomic epithelium; there is no early extraregional segregation and migration of germ cells. The undifferentiated condition of the gonad extends to about the end of the thirteenth day after insemination, at which time it is a compact mass of small cells with scattered large cells, the surface being an integral part of the mass, not separated as a distinct layer.

A second paper (Hargitt, '26) traced the history of the large and small cells of the gonad in the male from the beginning of sexual differentiation to sexual maturity. This covers the period from about fourteen days after insemination to forty-eight to fifty days after birth. In the male the large cells of the gonad completely disappear and the seminiferous tubules and all germ cells are derived from the small cells of the original genital ridge. The present report covers the history of the germ cells in the female from the time of sex differentiation to the first ovulation, that is, from about fourteen days after insemination to about forty-five days after birth. The same plan is followed as in earlier papers, viz., to describe the conditions at various stages objectively and follow this by discussion and interpretation.

MATERIALS AND METHODS

The material was gathered at the same time and treated in the same way as for the study of the male gonad, the animals being of the same strain. All young embryos were taken from the uteri, their membranes removed, and the body wall opened on the ventral side to allow the fixative to reach the gonads promptly. Older embryos were not only opened, but all the viscera, except the urinogenital organs, were removed completely, leaving these organs in place; thus the fixative was in direct contact with the gonads, which remained in place; the same methods were used for animals up to twenty-six days after birth. In animals twenty-six to forty-five days after birth the entire urinogenital complex was removed from the body and fixed separately. Preparations were always made rapidly, washed gently in a warm salt solution (Tyrode's), and placed at once in warm fixative. Fixation was in Bouin's fluid as modified by Ezra Allen (1½ per cent chromic acid and 2 per cent urea added) in the incubator at 37° to 39°C. for two and one-half to three hours, then gradually cooled to room temperature for thirty minutes to one hour, and the fixative replaced by the gradual addition of 70 per cent alcohol. The following stages were used: Embryonic stages: 14 days, 3 embryos; 15 days, 2 embryos; 16 days, 1 embryo; 16 days 18 hours, 5 embryos; 17 days, 2 embryos; one embryo each of, 17 days 20½ hours; 18 days 16 hours; 19 days 14 hours; 20 days 12 hours. Stages after birth: 2 animals of 3 days; 4 of 6 days; one animal of each of the following days, newborn, 9, 11, 14, 16, 19, 21, 23, 26, 29, 32, 36, 39, 43, 45 days after birth. In all, there are represented nine embryonic stages and seventeen postnatal stages. These were compared with ovaries of adults and old animals, a further study of which will be made later.

In this study, as in that of the male series, I have again been impressed with the necessity of having many stages which cover the entire period from the first appearance of the germ cells to sexual maturity. This is far more essential than to have many specimens of a few stages; any phase of the germ-cell history can be fully understood only if its relation to other phases is known. In the female series, investigators who might have embryonic stages or very young animals, and others just preceding puberty, would entirely miss the profound changes occurring between these periods and probably would come to an erroneous conclusion. Evaluation of the work of any investigator on such a problem as that of this paper must take into account the number of stages employed and the period covered, whether partial or complete.

OBSERVATIONS

1. History of the germ cells in embryonic stages

Embryo of fourteen days (fig. 1). In the male of this period the germinal epithelium began to be separated as a distinct layer—a condition which is not reached until much later in the female. Therefore one may distinguish a gonad which is developing into a testis, while there is no positive criterion for an ovary; if the organ does not show the characteristics of a testis, it is a developing ovary. As may be seen from figure 1, the ovary is a more or less indiscriminate mixture of large and small cells with no definitely differentiated stroma, the surface being an integral part of the mass; toward the center or deeper portion there is some tendency for the larger cells to group themselves into masses, but this is indistinct and does not involve the cells of the outer half. The figure shows a number of large cells arranged in a row, but this seems not to be characteristic. The large cells are somewhat more abundant back from the surface, but are fairly common in the surface also.

No cell walls are clearly defined at any point; the cytoplasm is about the same in appearance in all cells except the large ones; in these it sometimes stains a little deeper, while in others the stain is fainter. Nuclei of the surface layer are slightly elongated in form; some of the small nuclei deeper in the ovary are spherical and others elongated, those of the large cells tending toward spherical. In the nuclear network of all cells the chromatin is concentrated into several net-knots quite variable in number; perhaps the chromatin net of larger nuclei is slightly denser. There is nothing which permits one to think of a characteristic chromatin content or arrangement peculiar to one type of cell. Mitosis is common in all parts of the ovary, both surface and deeper, and this is true of cells of all sizes: it is more abundant in deeper cells, but common also in the surface. Only at the time of prophases or later nuclear changes does the chromatin stain deeply with nuclear dyes. A number of degenerate nuclei are found in different regions of the ovaries.

Embryo of fifteen days (fig. 2). There is a distinct tendency for the large cells deep in the ovary to clump together into groups of two or four cells; this is less evident near the surface (fig. 2). Some of the smaller nuclei are elongated and associated with large cells, probably the beginning of stroma formation. The large cells are sometimes clearly limited by a delicate bounding wall, but frequently this is lacking and, as in the earlier stages, the cytoplasm varies from very light staining to dark and more concentrated tinting with dyes. No change has occurred in the character and arrangement of nuclear contents. Mitosis is common in all kinds of cells both at the surface and deeper; the prophase stages are more abundant and may give a block-like appearance to the chromatin; telophases are also seen, but no metaphase figures could be found. Figure 2 shows telophases in surface and deeper cells, as well as one early prophase in a subsurface nucleus. This division partly accounts for the grouping of cells and also for variable size of cells. Both in the surface and deeper layers there is every gradation between the largest spherical nuclei and small oval nuclei; growth, divisions, transformations of size and form are occurring in the elements of the ovary; while large nuclei are dividing deep within the ovary, others are increasing in size; at the same time surface cells are dividing and others are growing to form large spherical cells as large as those embedded deeply in the ovary. Degeneration of nuclei occurs sparingly and only toward the base of the ovary.

Embryo of sixteen days (fig. 3). As in previous stages, no cords of cells are present and the cells and nuclei of different sizes are well distributed, though large nuclei are more abundant near the surface. Cell walls cannot be distinguished and the cytoplasmic mass shows little difference in density, arrangement, or staining reaction in any portion of the gonad; neither is there any general or marked difference in the appearance and staining of chromatin in nuclei of different form and size. Mitosis is very common in the surface layer and in the spherical nuclei of smaller size within the deeper part of the ovary; the large spherical nuclei frequently show solid chromatin masses or strands suggestive of prophase conditions, but very few metaphase or telophase stages are encountered. The large nuclei are not more abundant than in the ovary of a fifteen-day embryo, but spherical nuclei of somewhat smaller size, with similar structure and appearance, are suggestive that division has occurred. The surface layer contains some spherical nuclei as large as those deeper in the ovary, and intermediates in size and form occur superficially and deeper. Below the surface many degenerate nuclei are found which seem to represent the larger spherical cells.

Embryo of seventeen days (figs. 4 to 6). Near the dorsal part of the ovary where it is attached to the body wall, a delicate tunica albuginea is present with some stroma between the egg nests (fig. 6); elsewhere the surface layer is a part of the ovarian mass, with only a few elongated nuclei foreshadowing the formation of stroma (figs. 4, 5). Except where an albuginea is present, the cells of the surface layer do not differ from deeper cells. Large cells are now distinctly grouped into nests surrounded by very delicate membrane-like fibers (figs. 4 to 6), such nests being more definitely marked at the base (figs. 5, 6) than near the free edges (fig. 4). In some nests cell outlines can be clearly distinguished (fig. 6), in others very indistinctly (fig. 4), and in still others none can be found (fig. 5). In general, the cytoplasm of cells throughout the ovary is uniform, possibly a bit vacuolated in some cells, but not differentiated into light- and deep-staining cell masses. The figures demonstrate that the cells which are forming nests are of two sizes; in both the nuclei are spherical, but some are larger than others; in most ovaries the smaller nuclei are more abundant, but occasionally the large exceed them slightly. There are intermediate conditions between these two types, as well as between irregular and spherical nuclei, and between much smaller and very large ones. The chromatin of the largest nuclei is frequently in small deeply staining granules with several nucleoli; that in the small spherical nuclei is more commonly in block-like masses, but since this is not constant it is doubtful whether it is a typical or significant difference.

Mitosis is common throughout the ovary; of the cells in nests it occurs more frequently in the smaller nuclei; these show more metaphases, anaphases, and telophases, while the larger nuclei more commonly are in prophase stages, with few later ones to be observed. This is a more or less general difference, though it varies in different ovaries and is not constant in any. Mitosis also occurs commonly in the surface layer. Figure 4 demonstrates clearly that some nuclei of the surface layer are as large as the largest nuclei in nests, and later embryonic stages will enable one to follow such cells into the formation of nests. Since all sorts of intermediates are found in the surface, both in form and in size, it is clear that this surface layer is contributing to the formation of different elements of the ovary. A condition not infrequently noted is that illustrated by figure 5, namely, that both large and small nuclei of the nests may undergo degeneration; this occurs more often in deep than in superficial nests.

Embryo of seventeen days seventeen hours (figs. 7 to 11). The presence of a delicate hair-line or membrane beneath the surface layer (fig. 7) represents the early formation of a fibrous tunic, but it does not completely enclose the ovary; along the ventral border the surface and deeper layers are still merged. The only stroma or fibrous tissue within the ovary is found in delicate layers about each cell nest (figs. 7, 11); the nuclei of this tissue are much elongated like connective-tissue nuclei. A characteristic of this period is the occurrence of cell nests throughout the entire ovary, each with delicate surrounding stroma. As shown in figures 7 and 11, most cells within the nests are delimited by delicate cell walls, discernible only under oil-immersion lenses. The cytoplasm of all cells, whether in nests or surface layer, has the same appearance; only at the time of mitosis when the nuclear membrane has ruptured does the cytoplasm differ by assuming a lightly staining, alveolar appearance. Figures 7 and 11 are parts of two nests, the former from near the edge, the latter from the basal portion of the ovary. In each nest three types of nuclei may be distinguished: irregular or elongated which will later form the follicle cells, large and smaller spherical nuclei. Some of the largest spherical nuclei possess delicate strands of chromatin (figs. 8, 9) which resemble the leptotene condition, while in others these strands are denser, are massed against one side of the nuclear wall (fig. 10), and seem to mark the beginning of the synizesis stage. Some of the synizesis stages end in degeneration, but most pass on to later phases of development. The smaller spherical nuclei are less abundant, their chromatin is frequently block-like, or else definite chromosomes are present in a clumped metaphase plate (fig. 11), but anaphase and telophase stages are rare. There are intermediate stages between separate chromosomes, clumped chromosomes, early degeneration, and late degeneration. Possibly the smaller spherical nuclei do not complete their division and end in degeneration, but this is not at all certain; nor is it clear that the large and small spherical nuclei represent different types of cells. Comparison of large and small nuclei in figures 7 and 11 indicates that leptotene and synizesis stages are found in both.

This is the earliest embryo in which meiotic phenomena were seen. However, the initiation of meiosis does not inhibit the growth and development of large cells in the surface layer; figure 7, from the ventral border of the ovary, shows the surface epithelium to consist of cells with ovoidal nuclei, but with the cells rather large; in this section two surface cells have enormously enlarged and are beginning to press into the ovarian mass. In none of the cells in such position does the chromatin exhibit leptotene conditions, but later stages indicate that these cells become primary follicles; consequently, the surface is still contributing to the formation of germ cells. Mitosis of cells in the surface layer is less common than formerly, but still occurs.

Embryo of eighteen days seventeen hours (figs. 12 to 14). In the ovaries of this period there is a germinal epithelium of two or three layers separated by an extremely delicate fibrous layer, the beginning of the tunica albuginea, which nearly encircles the ovary, but has not formed at the extreme ventral edge where the surface is directly continuous with deeper tissues. A similar delicate stroma surrounds each nest of cells; in both places the fibrous tissue contains elongated nuclei. Nests of cells completely fill the ovary, extending from the base nearly to the free edge; occasionally even groups of two or three cells in the germinal epithelium are enclosed by delicate fibers. Cell walls are not so clear as formerly and are quite unrecognizable in the germinal epithelium. There is little, if any, differentiation of the cytoplasm in cells of different sorts and in different places, the cytoplasm of the entire ovary is about the same in its appearance and staining reaction. Figure 12 is a drawing which is typical for the cell nests of this stage: the large nuclei of the nests are in stages of contraction or synizesis, some show leptotene threads, others are entering the synizesis condition, and others are yet more contracted. The chromatin strands are in loops massed at one side of the nucleus (fig. 13) and appear single; in a very few there may be a possible hint of a double thread, but it is too uncertain to demonstrate positively; in others the chromatin is condensed into a mass in which threads can be made out only with difficulty (fig. 14). Nearly all nuclei of the nests are in the same condition, whether they happen to be large or small; the smaller nuclei of the previous stages which were usually in stages of mitosis are uncommon, though some occur. This would seem to indicate that the two apparent cell types of earlier stages represent the same thing, and do not constitute different categories. Mitosis is quite common in the germinal epithelium, both in surface and deeper layers; the axis of the spindle varies from parallel to perpendicular to the surface; the nuclei of this layer are generally ovoidal, but some are spherical, much larger, and with heavier chromatin; some of these large cells grow into germ cells which remain for a time superficially located, but finally get below the surface. Those deep in the germinal epithelium, even when isolated, may show leptotene and synizesis stages of chromatin, though not when in the surface layer. Synizesis seems almost universal in the large cells of this stage, whether they have been present for some time or have just developed. As figure 12 shows, degeneration of nuclei occurs and the condition of the degenerating nuclei gives evidence that this may overtake them in the stage of mitosis or of synizesis and contraction.

Embryo of nineteen days fourteen hours (fig. 15). Conditions in embryos of this period differ but little from those of the preceding one, as a comparison of the drawings (figs. 12 and 15) will demonstrate. The threads in the nuclei of the nests are more granular, wider, not so definitely looped, and are less crowded; they appear single and more tangled than previously. Degenerating nuclei are less common, but present both in nests and in large cells within the germinal epithelium. Mitosis is common in the germinal epithelium, and the large cells with spherical nuclei containing granular chromatin are present both in the surface and in the deeper layers, and immediately beneath it. These germinal epithelial cells seem always to enlarge singly or in small groups and never in the form of cords, nor have cords been present at any time in the development of the ovary.

Embryo of twenty days twelve hours (figs. 16, 17). The peritoneum has by this time almost completely enclosed the ovary within an ovarian capsule. The germinal epithelium varies from one to four cells in thickness and a delicate tunica albuginea is present everywhere beneath the germinal epithelium; the stroma is more abundant and each nest of large cells is completely enclosed within a delicate fibrous tunic. The cell nests extend radially from near the surface to the depths of the ovary constituting well-marked medullary cords, but not separated from superficial cortical groups; the cord-like appearance is due to rearrangement of cells and nests, the developing germ cells do not arise in the form of cords in any embryonic stage. In places the germinal epithelium is much thicker and the tunica albuginea extends into it, cutting out new groups of cells. Within the nest the larger cells are being surrounded by elongated nuclei to form the follicular epithelium; no significant differences in the cytoplasm are found in the different parts of the ovary. Mitoses are taking place in the germinal epithelium, but not in abundance, and most nuclei are small and ovoidal with only an occasional enlarged spherical one. However, as shown in figure 16, there are places where the germinal epithelial cells have enlarged to form germ cells.

The chromatin of the large nuclei is in the form of loosely arranged strands or loops, with a fairly clear polar arrangement. These strands are wider and heavier than in previous stages and the large granules give a moniliform appearance (fig. 17). Since no evidence of a double or split thread occurs, this would seem to correspond to the pachytene stage. The characteristic chromatin arrangement is found in the nuclei of large cells deep in the ovary and also in those near, or even within, the germinal epithelium (fig. 16).

2. History of the germ cells during the postnatal period

Newborn animal (figs. 18, 19). There is little difference between this and the last embryonic stage in the general relation of the ovary and its parts. In some nests primary follicles are definitely established, though this is by no means universal, even in neighboring cells (fig. 18). The chromatin of the germ cells is less deeply staining, though it is still strand- or loop-like with no definite evidence of a diplotene condition (fig. 19). These chromatin strands begin to show irregular mossy extensions along the edges and concentrations of chromatin into nodes—the beginning of the resting condition of the nucleus. Cells of nests in various portions of the ovary exhibit degeneration phenomena (fig. 18), as was also true in the last embryonic stage.

Three days after birth (figs. 20 to 23). The germinal epithelium is two to three cells deep, except near the hilus where there is a tendency toward a single row of cubic cells. The tunica albuginea is loose and delicate, with few fibers. The nests of large cells so characteristic of earlier stages have broken up and the ovary now is completely filled with a mass of primitive follicles separated by loose stroma; the number of these primitive follicles seems much smaller than the number of large cells present in nests at birth and just before. The nuclei of the ova now rarely display any polar or loop-like arrangement of chromatin strands, though occasional remnants of this may be recognized (figs. 20, 21); for the most part the chromatin is in a network characteristic of resting nuclei (figs. 20, 23). At no time has there been any clearly marked diplotene stage, though figure 22 shows some slight evidence of this.

Figure 20 indicates very clearly that the germinal epithelium is still active in proliferating cells which enlarge and push through the albuginea into the ovary, the deeper ones of these being possessed of follicle cells. In figure 21 there is a single enlarged surface cell which resembles the oocytes within the ovary—in this case the chromatin is in a leptotene or pachytene condition. In general, the nuclei of the germinal epithelium are small and ovoidal, but a considerable number are spherical and larger, with denser, more granular chromatin, and there are a considerable number of very large cells with spherical nuclei which do not differ from the large nested cells of earlier stages. These cells of the germinal epithelium show intermediates with such large cells as the one represented in figure 21. There is some degeneration of cells and nuclei, but not greater in amount than in previous stages, and certainly not abundant enough to account for the apparent decrease in the large cells which were present at an earlier stage.

Six days after birth (figs. 24, 25). The germinal epithelium is two or three cells thick, rarely single-layered, and in some places five or six cells thick; the tunica albuginea is slightly thicker, but still delicate. The ovary, like that of three days, is filled with primitive follicles, those near the hilus being enlarged and the follicular envelope composed of several rows of cells. There are masses of oocytes crowded beneath, and partly in, the tunica albuginea (fig. 24) either completely naked or possessing only a few scattered follicle cells; this group of cells has developed since the three-day stage. In addition, the germinal epithelium has more cells with spherical nuclei, some of which are greatly enlarged and like the oocytes within the ovary. The single large cell of the germinal epithelium represented in figure 25 is without accompanying smaller cells, but in other places small follicle cells are surrounding these isolated cells while in the surface layer. Also, figures 24 and 25 show enlarged oocytes just beneath the surface within the tunica albuginea. Considering the large cells in the germinal epithelium and the masses of oocytes just below, it seems clear that renewed contributions are being made from the surface layer to the oocyte content of the ovary.

All the nuclei of the germ cells are in the resting condition (figs. 24, 25), with no evidence of pachytene, diplotene, or loop-like threads of chromatin. In the three-day animal large cells in the germinal epithelium and below often had pachytene chromatin threads, but in the six-day individuals the newly enlarged cells show none of these meiotic phases; between the three- and six-day stages all evidences of meiosis are lost and germ cells produced from the surface after this do not display such conditions. Little evidence of degeneration of cells or follicles is found, though the number of follicles has decreased.

Nine days after birth (fig. 26). The ovary of this stage agrees with that of the six-day animal in that the deeper part is filled with primitive and young follicles, while near the surface are masses of oocytes none of which are completely surrounded by follicle cells. Unlike the six-day ovary, enlarged cells and oocytes could not be found in the germinal epithelium. A noteworthy feature, seen for the first time, is the presence of cords of cells extending from the germinal epithelium into, or through, the tunica albuginea, as represented in figure 26. Other similar cords are found parallel to the surface, but not apparently connected to it. The nuclei of these cords are usually spherical and considerably larger than those of the germinal epithelium; furthermore, some of the cords terminate in deeper pockets containing oocytes which may be partly surrounded by follicle cells. The total number of follicles in the ovary has decreased without any direct evidence of profound degeneration.

Eleven days after birth (fig. 27). This ovary closely resembles that of the nine-day animal, with a few of the deeper follicles becoming vesicular. The masses of oocytes near the surface in earlier stages have broken up and are becoming primitive follicles. Mitosis is more common in the germinal epithelium, but the cord-like proliferations of nine-day ovaries do not show; on the other hand, single enlarging cells in this surface layer are more common. The germinal epithelium is becoming single-layered and frequently flattened; the occurrence of enlarged cells is, therefore, more easily noted. For example, figure 27 shows one enlarged surface cell and another, much larger, just beneath the surface surrounded by smaller cells, not in follicular form.

Fourteen and sixteen days after birth. The ovaries of animals of these ages are in general characterized by the following features: 1) the ovary is filled with follicles practically all of the same size and most are becoming vesicular, these follicles extend nearly to the surface; 2) just beneath the tunica albuginea are many oocytes each with a delicate follicle surrounding it and reminding one of so-called 'residual ova' or oocytes of adult ovaries; 3) still closer to the surface are other oocytes without surrounding follicle cells, but otherwise similar to the oocytes with follicles, both in size and in the character of the nuclei. The germinal epithelium is one or two cells thick, but tending to become single-layered and flattened; mitosis occurs commonly, but cord-like ingrowths are rare and only a few ovum-like large cells could be discovered.

Nineteen days after birth (figs. 28 to 31). The most striking character of these ovaries is the great growth of some of the follicles into graafian follicles which have moved close to the surface, and the absence of any large number of oocytes and primitive follicles. The stroma has increased in amount and becomes much looser and the follicles are not so closely packed. Anovular follicles or follicles with degenerate or fragmented ova were not observed. The germinal epithelium has become flattened and mesothelial-like, with frequent thickened patches in which are located single, enlarged, ovum-like cells which seem to have formed in place. Figure 28 shows one such enlarged cell with a large spherical nucleus in the single-layered surface epithelium of the ovary; figure 29 represents another which is being surrounded by follicle cells; in figure 30 a similar cell is completely surrounded by small cells; and figure 31 demonstrates the production of a follicle about the large cell and the pressing of the whole into the tunica albuginea. While there are relatively few of these cells in ovaries of this stage, their presence indicates a continued activity of the germinal epithelium in contributing to the number of germ cells of the ovary. Both near the hilus of the ovary and at other points are cord- or tube-like masses of epithelial cells often connected with the surface and at other times parallel with this, but in the tunica albuginea. In the region of the hilus these are distinctly tubular and resemble rete tubes; no enlarged cells are present. It may be suggested that somewhat similar cord-like structures on the opposite side of the ovary are likewise not correlated with germ-cell formation; there is some evidence in favor of this view, but in a number of cases cells of these cords are larger and may even contain much enlarged spherical cells which do not differ from isolated oocytes within the ovary.

Twenty-one days after birth. The ovaries of this animal resemble those of nineteen days, since they are completely filled with large growing follicles, many of which are vesicular and some degenerating. In this stage it becomes clear that the great majority of all ova in the ovary are in active growth, for the number of primitive follicles and oocytes is far below that of the fourteen- and sixteen-day ovaries and the total number of ova in all stages seems to be less than in six and nine days. Oocytes and very young follicles are so few that they are observed only near the surface in the region of the hilus, where they are fairly common. The germinal epithelium is mostly flattened and single-layered and shows little indication of activity in producing large cells.

Twenty-three days after birth. In one respect this ovary is like that of twenty-one days, namely, that the entire ovary is almost completely filled with large graafian follicles and a very small number of oocytes and primitive follicles. The large graafian follicles, whether at the surface or deeper in the ovary, have reached the limit of their growth and are forming the first polar body, or the first maturation spindle. This process was begun slightly earlier than twenty-three days, for many graafian follicles in the center of the ovary show fragmented ova undergoing degeneration; there are other graafian follicles with a germinal vesicle not quite ready for polar-body formation. So few are the oocytes and primitive follicles that one may say practically all ova in the ovary are near the end of the growth period and practically all germ cells present in ovaries at birth and shortly after have gone through an apparently steady and normal growth at the same time, producing an ovary of mature follicles and almost no reserve of younger stages. With this is correlated another fact, that the germinal epithelium is the least active it has been at any time in its history; there are fewer enlarged cells in the surface and fewer oocytes in or beneath the tunica albuginea and these are widely scattered.

Twenty-six and twenty-nine days after birth (figs. 32 to 34). The general appearance of the ovary is similar to that of twenty-three days; the greater number of graafian follicles are near the end of growth, some with germinal vesicles, others with maturation spindles, and still others with the first polar body completely formed. Large numbers of degenerate follicles are present, some with fragmented ova, others with little of the ovum remaining, and, especially in the twenty-nine-day stage, many which have practically disappeared, leaving only a few masses of granulosa or theca cells; these degenerate follicles are more abundant near the center of the ovary. There are more oocytes and primitive follicles, especially in twenty-nine days, than there have been since the early days after birth. The naked oocytes and smaller follicles are just below the germinal epithelium in the tunica albuginea and the primitive follicles are in or below the albuginea; none of these young stages are found far from the surface of the ovary. The germinal epithelium is flattened, with groups of cubic cells at different places. Not many mitoses occur, but it is common to find single cells enlarged and bulging outward, each having a large spherical nucleus. In figure 34 one such cell is shown, only slightly enlarged, while a similar cell in figure 32 is greatly enlarged and associated with a group of smaller cells; figure 34 also shows a group of large cells below the surface cell, as though this region had recently formed several large cells which had sunk into the tunica albuginea. Such clusters or pockets of cells as are represented in figure 32 are common, sometimes with one large cell, often with none. Another common occurrence is figured in 33, a long cord of cells larger than germinal epithelial cells frequently with one or more large cells undistinguishable from oocytes or the ova of young follicles. None of the large cells, whether in the surface layer, tunica albuginea, or below, show any evidences of leptotene or other meiotic phases; in all these locations the nuclei are like the usual resting nucleus. The enlargement of cells in the surface does not proceed very far when they begin to sink into the ovary, hence these earlier stages are easily overlooked and often are more abundant than at first appears.

Thirty-two days after birth. The center of the ovary is filled with much loose stroma or connective tissue with scattered remnants of degenerated follicles. Outside this region and close to the surface of the ovary are a very few graafian follicles, the ova of which are in maturation, or more commonly in fragmentation; a very few show a germinal vesicle. Smaller young vesicular follicles are present, some solid follicles, with primitive follicles and oocytes. Large cells are scarce in the surface layer.

Thirty-six days after birth (figs. 35 to 38). Again at this stage the ovary is filled with follicles; most of these are large graafian follicles, some with the ovarian nucleus in the germinal-vesicle stage, others in maturation mitoses, and many are degenerating with scattered chromosomes, several nuclear bodies, or fragmented ova. Most of the earlier degenerate follicles have been completely absorbed, leaving, at most, a few remnants toward the center of the ovary. Primitive follicles are abundant, some of them starting to grow, and oocytes are numerous, mostly opposite the hilus, but others scattered elsewhere. The germinal epithelium is more active in forming ingrowths and large cells than it has been since the embryonic period. Two types of activity occur: the less common is shown in figures 35 and 36, the germinal epithelium thickens to form pockets of cells (fig. 35) which extend into the tunica albuginea; these may be cut off as groups, while others form elongated cords (fig. 36) which later separate from the surface. While these cords often contain cells not different from surface cells, not infrequently enlargement of certain cells may occur and ultimately distinct oocyte-like large cells are produced like those figured in 36; these do not differ from oocytes or young follicles shown in figure 35. The more common type is the enlargement of single germinal epithelial cells; figure 37 represents the growth of a single cell at the surface, its nucleus is spherical and the cell is being surrounded by follicle cells; this development leads to the formation of primitive follicles either while still in the surface (fig. 38) or not until the cell reaches the tunica albuginea. In ovaries of this stage such transformations are common and many germinal epithelial cells exhibit various phases of enlargement and passage into the ovary. Such cells never pass through any of the phases of meiosis.

Thirty-nine days after birth. This ovary resembles the previous one in the large number of degenerating follicles, the large graafian follicles with maturation spindles, and of smaller follicles near the surface. The number of oocytes and young follicles may be somewhat greater, though it is difficult to estimate closely. There is a difference from the earlier stage in that the germinal epithelium does not present the evidence of great activity found in thirty-six days.

Forty-five days after birth (fig. 39). At this time ovulation has occurred and seven large corpora lutea are present in a single ovary; ova could not be discovered in the fallopian tubes or upper end of the uterus. Actual counts of large follicles in a single ovary were made: at least thirty-nine follicles were at the surface apparently ready for discharge, the ova of which contained resting nuclei, maturation spindles, or polar bodies; there were over fifty large follicles showing very early stages of degeneration, and near the center of the ovary were very great numbers of remnants of follicles in late stages of degeneration. In this and other earlier ovaries, primary and graafian follicles may begin to degenerate before reaching large size and while still deep within the ovary; the degeneration of neither the large nor small follicles seems to result in the formation of any corpora lutea or even of luteal cells. Considerable numbers of growing follicles are present, a small number just beginning to grow, and numerous primary follicles and oocytes, the latter always close to the surface of the ovary.

In the germinal epithelium are found somewhat enlarged spherical cells which protrude from the surface and many more which are already partly surrounded by small follicle cells. Some are pushing from the surface layer into the albuginea (fig. 39). Many oocytes just within the edge of the ovary give the impression of having recently come from the surface epithelium. Another animal of a day or two earlier showed practically the same condition of its ovaries; therefore between forty and forty-five days seems to be about the time, in this strain of albino rats, in which normal ovulation occurs and in which sexual maturity has been reached.

3. *Summary of observations*

While the testis can be positively identified in embryos of fourteen days by the separation of a definite surface layer of the gonad and the formation of a tunica albuginea, the ovary remains as a solid mass with no such distinction until about the time of birth. In embryos from fourteen to sixteen days the ovary is composed of large and small cells intermingled without definite order, both at the surface and deeper in the ovary. In form and size gradations occur between cells of various sizes and no specific differences can be found either in the cytoplasm or nuclei; mitosis occurs both in large and small cells. At seventeen days the large cells are definitely grouped into nests separated by delicate stroma tissue, some cells of the surface layer are as large as, and similar to, those in the nests, and gradations occur between large and small surface cells. In eighteen-day ovaries meiotic phenomena are seen for the first time, the cells beginning to show leptotene and synizesis stages. During the nineteenth day the cell nests completely fill the ovary and all spherical nuclei are in stages of leptotene, synizesis, and contraction, also isolated cells in the germinal epithelium possess leptotene threads. In embryos of the twentieth day the ovary becomes almost completely enclosed within a peritoneal capsule and a tunica albuginea forms as a very delicate layer completely separating the germinal epithelium. The cell nests have a radial arrangement like medullary cords, though this is due to rearrangement of nests, for no cords are formed in embryonic stages of development. Large cells are being surrounded by follicle cells, and ovarian nuclei now display the pachytene condition. At birth there is little difference except for the completion of a follicle layer about some ova and the production of the resting condition in the chromatin of the nuclei.

Three days after birth, the nests of germ cells break up and the ovary is filled with primitive follicles, the number of which seems smaller than the number of ova in nests of previous stages. The nuclei of germ cells are leaving the pachytene stage and most are in the resting condition, though a few large cells in the germinal epithelium show leptotene and pachytene forms; this is the last stage in which meiosis shows in the ovary. By six days after birth some of the deeper-lying ova have a several-layered follicle; at this stage, also, masses of naked oocytes are found in, or just below, the tunica albuginea, apparently having been produced since the three-day period; these show no meiosis. The germinal epithelium contains many enlarged cells, some with, and others without, follicle cells. In ovaries of animals nine and eleven days after birth some deeper follicles are becoming vesicular and the naked oocytes seen in three and six days have become surrounded by a single layer of follicle cells. A few enlarged cells are present in the surface layer and this tissue has proliferated into masses of small cords extending into or through the tunica albuginea; some of the cells of the cord cannot be distinguished from oocytes. From eleven up to sixteen days the older follicles become graafian follicles so that the ovary is filled with them. Again during this period oocytes have accumulated just beneath the surface, most of them being naked, but some with a few follicle cells surrounding them. At sixteen days the germinal epithelium has become single-layered and mesothelial-like, it contains no enlarged cells and is forming no ingrowing cords. By nineteen days the graafian follicles have moved close to the surface of the ovary, below this small follicles are growing, while primitive follicles and oocytes are not common; the surface layer of epithelium contains a few enlarged cells and some cord-like ingrowths.

In animals twenty-three days old the ova and follicles seem to have reached the end of their growth, the ovary is filled with graafian follicles many of which are forming the first polar body, and many follicles with fragmented and degenerating ova are massed in the center of the ovary. Oocytes and primitive follicles are fewer than at any former period and the germinal epithelium is the least active in producing large cells that it has been at any time during the life of the animal.

During the twenty-six- to twenty-nine-day period practically all the graafian follicles reach their maturity, for more and more of their ova form maturation spindles and polar bodies, until finally at thirty-two days only a few graafian follicles remain at the surface with the ova in maturation. Paralleling this series of changes there is a progressive increase in the number of degenerating follicles, so that the center of the ovary becomes a mass of remnants of degenerating follicles with fragmenting ova which gradually are absorbed; ultimately at about thirty-six days there are left only small concentric masses of follicular tissue scattered through the center of the ovary. There is a third series of concomitant changes during this period; oocytes, primitive follicles, and activity of the germinal epithelium decrease through fourteen, sixteen, and twenty-one days and reach their lowest ebb at twenty-three days. In ovaries of twenty-six and twenty-nine days increased number of oocytes and primitive follicles are found and many large cells form in the germinal epithelium and move into the ovary. While not constant or steady, there is a continued increase, and in thirty-six- and thirty-nine-day ovaries the germinal epithelium is more active in forming large cells than it has been at any time since early embryonic stages. The increase in the production of large cells or oocytes in the ovary, just summarized, is followed by renewed growth phenomena; by thirty-six and thirty-nine days the ovary is again filled with large graafian follicles some of whose ova are in the process of maturation, also there are numerous stages in growth of follicles more recently produced, and numbers of ova in stages of early degeneration within mature follicles. Thus there is a renewal of the previous cycle, namely, formation of oocytes, follicle formation and growth, maturation, and finally degeneration of the ova and their absorption along with the follicular tissue.

About the age of forty-five days ovulation occurs for the first time and the ovary may be assumed to be mature. But the first ovulation is not quite typical, perhaps, of the later rhythmically recurring ovulations. For example, in a single ovary of this age were counted seven large corpora lutea, thirty-nine follicles at the surface almost ready for ovulation, over fifty follicles in early degeneration and a very large number of remnants of follicles in late phases of degeneration; the ovary of this period gives the impression of finally ridding itself of all growing follicles and ova at the time of the first ovulation. In this ovary of forty-five days there are a small number of growing follicles of younger stages, more primitive follicles, and numerous oocytes. Within the germinal epithelium are large spherical cells, and others, like young oocytes, are passing into and through the tunica albuginea.

DISCUSSION

An analysis of the abundant literature on mammalian germ cells shows two things: 1) Observations similar to those recorded in the previous section have been made by other investigators on the rat, mouse, cat, dog, rabbit, human, and other mammals; it seems clear that nearly all workers have seen the same things and would agree upon the objective facts. 2) Certain observations seem important to some and incidental to others; the same facts are explained differently; various opinions are expressed as to the probable course of events. The diversity is largely one of interpretation and not of observation. The chief questions at issue are these: Are germ cells a special type of cell or merely transformed tissue cells; do they originate in the gonad or migrate into it; do the definitive ova represent a persistence of the first cells formed in the gonad or are they newly developed; are distinguishable meiotic phenomena necessary criteria of germ cells?

The germ cells are not a special type of cell nor do they migrate into the gonad from some outside source; rather the entire gonad and its various constituent cells are produced by proliferation of the coelomic epithelium. The genital ridge when first distinguishable as an indifferent gonad is a rather compact mass of cells not separated into layers and regions, but containing large cells indifferently scattered among the more numerous small cells, both of which have the same origin. The large cells have usually been called primordial germ cells; their origin and significance are discussed in an earlier paper (Hargitt, '25).

1. The germ cells in the embryonic ovary

The indifferent gonad just before sexual differentiation is a mixture of large cells and far more numerous small cells. In the testis the large cells increase by division and become more numerous, but they ultimately degenerate and disappear; the large cells of the ovary may persist and form follicles later, they also increase greatly in numbers. This increase of young ova is partly by mitotic division (figs. 1, 2, 5, 6) which ends in the gonad of the eighteen-day embryo, but others are produced from the surface layer of the gonad (figs. 1, 2, 3, 7, 16). Rubaschkin ('09, '12), in the mouse, dog, cat, pig, and guinea-pig, Fuss ('13), in man, pig, and dog, League and Hartman ('25), in the opossum, and Cowperthwaite ('25), in the albino rat, state that either there are no large cells present in the germinal epithelium or else, while present, they do not form ova. On the other hand, Foulis ('76), Balfour ('76), Schulin ('81), Harz ('83), Nagel ('88, '89), Bonnet ('91), Schottländer ('93), Bühler ('94), Coert ('98), Skrobansky ('03), B. M. Allen ('04), Felix ('06, '12), Winiwarter and Sainmont ('08), Kingsbury ('14), Kirkham ('16), Kingery ('17), Firket ('20), van Beek ('24), Butcher ('27), Brambell ('27), and Simkins ('28), in the cat, dog, pig, cow, man, rabbit, fox, mouse, rat, are all convinced that the cells of the germinal epithelium of the embryonic ovary do transform into ova. Rubaschkin also states that the mitochondria of germ cells are always granular in form, while all other cells possess rod-shaped mitochondria. This was accepted by Tschaschin ('10) for the chick, Fuss ('13) for man, pig, and dog, and Rauh ('26) for the rat; but Meves ('07), after a very careful study of mitochondria, concluded all cells of the embryo, from all three germ layers, have identical mitochondrial bodies, and there is no possible distinction of germ cells on this basis. This conclusion for the chick, mouse, and guinea-pig was confirmed by Kingery ('17) in the mouse, and Firket ('20) in the chick and rat; also, others working on the germ cells of mammals have been unable to confirm differences in mitochondrial elements of germ cells and tissue cells; likewise, Kulesch ('14) discovered that Golgi bodies were alike in all cells of the ovary. From this great preponderance of opinion we must conclude that, during the embryonic period, the ovary produces germ cells by a transformation of some of its germinal epithelial cells, and these transformed cells are identical in form, size, structure, and fate with any other germ cells. Nagel and Coert go further in stating that the peritoneum outside the ovary can also produce identical young ova. From my own observations it seems clear that there are relatively few germ cells in the undifferentiated gonad and that the vast majority of the germ cells have been produced by transformations of the germinal epithelium of the embryonic ovary after sexual differentiation; this process is a continuous one throughout the embryonic period and is also widespread after birth.

Formerly, there was a good deal of discussion as to whether the germinal epithelium formed ova alone, follicle cells only, or both; at present there is practically an agreement that both ova and follicle cells come from this layer, though League and Hartman ('25) think in the opossum only follicle cells can be so produced. Conditions like those shown in figures 1, 2, 3, 7, 16 of the present paper show definitely that the surface of the embryonic ovary does actually produce young ova, especially since such cells may be followed directly into the production of follicles containing ova. The statements of a few authors, as Rubaschkin and Fuss, that large cells present in the surface layer have migrated into this position fail to take into account two facts: First, that there is little evidence of actual migration and, further, that the enlargement of such cells occurs first in the epithelial layer itself and only later are they found in the deeper part of the ovary. Secondly, and more significant, is the fact that transitions between the ordinary germinal epithelial cells and the large germ cells in the ovary can easily be demonstrated, the intermediate stages are so clear that there can be no doubt of an actual transformation. Brambell ('27) is convinced of this transformation in the mouse and shows further that some of these germ cells may be pushed *out* of the gonad to form the extraregional primordial germ cells. The extraregional germ cells, therefore, also originate in the gonad.

The ova, at first scattered, become grouped into nests about seventeen days after insemination and remain so until shortly after birth (figs. 4, 5, 6, 7, 12, 15, 18); at first the ova are naked, but gradually become enclosed within follicles and surrounded by connective-tissue sheaths. As already stated, there is a continuous process of formation of ova from the germinal epithelium and the production of new nests; the result is that just before birth the ovary is a mass of closely packed egg nests. During this process there is more or less degeneration of ova within the nests (figs. 11, 12, 15, 18), but the new production of eggs is greater than the degeneration, so the embryonic period is distinctly one of increase in numbers of ova. A widespread degeneration occurs after birth and will be discussed later.

Meiosis occurs first in the ova of the eighteen-day embryo and is completed three or four days after birth. The leptotene condition shows during the eighteenth day (figs. 7, 11), the synizesis and contraction in the nineteenth (figs. 12, 15), and the pachytene about the twentieth day (figs. 16, 17); the diplotene condition could not be recognized at any stage. While these changes occur first in ova within nests, by the twentieth day similar large cells in the germinal epithelium display the same phases (figs. 16, 21). For those who maintain that the germinal epithelium cannot produce ova figure 21 should be illuminating: here is a cell formed by enlargement of a germinal epithelial cell in the pachytene stage of meiosis. Pratt and Long ('17) observed meiosis beginning in the seventeen-day embryo rat and ending two or three days after birth, Rauh ('26) found the leptotene on the seventeenth day, Cowperthwaite ('25) discovered no meiosis later than three days after birth, Butcher ('27) noted the pachytene condition at birth and diplotene on the third day after, Brambell ('27, '28) could discover no meiosis later than four days after birth. Therefore there can be little doubt that the general meiotic phenomena come between the seventeen- or the eighteen-day embryo and the period of three to four days after birth. The possibility of later meiosis and the significance of the process will be discussed later.

2. Postnatal history of the ova

The ova which are produced in large numbers in the embryonic ovary group into nests; about three days after birth these break up and the ovary is completely filled with primitive follicles. So far as one can tell all these begin to grow at the same time, and by twenty-six to twenty-nine days after birth the maximum size is reached and maturation is in progress; this is about ended by thirty-two days. Degeneration of large follicles is first noticeable about twenty-one days after birth and by thirty-six is almost completed; this degeneration involves all the follicles which started on their growth a few days after birth. Thus it appears that the ova present a few days after birth go through a normal growth and maturation process and then all degenerate, first the ova and then the follicles. Following this, from thirty-six to thirty-nine days, the ovary again becomes filled with large graafian follicles and many of them go through a similar maturation and degeneration; probably some of these, later in developing, persist until the first ovulation at forty-five days. The first epidemic of growth and degeneration, it seems to me, involves those ova formed mostly during the embryonic period and present a few days after birth, while the second, less extensive, degeneration is of new ova which have been produced after birth. Such an extensive growth and degeneration in immature mammalian ovaries is probably of general occurrence. In the mouse Lange ('96) states that at twenty-eight days all the ova have grown to perfect follicles, all at the same time, and the greater part of these degenerate; Kirkham ('16) finds that between seventeen and forty days a great many ova form perfect follicles and degenerate; Kingery ('17), that all germ cells present at birth grow, mature, and finally degenerate by absorption or atresia between twenty and twenty-five days, all at the same time; Brambell ('27) notes what he calls a 'precocious growth' of large numbers of ova, all of which degenerate about the time of weaning, about twenty-one days after birth. In the cat Kingsbury ('14) records a growth of many ova and a profound degeneration before sexual maturity. In the rat Arai ('20) believes all the primary germ cells of the ovary degenerate, mostly after growth into large follicles, most extensively and rapidly between twenty-one and twenty-three days; Butcher ('27) completely confirms Kingery (for mouse) that all embryonic germ cells degenerate, mostly by follicular degeneration, beginning about thirteen days and ending between twenty and twenty-four days. In the dog Schotterer ('28) finds that the vast majority of all ova disappear through atresia in the first few weeks of life. In man Simkins ('28) finds all the large cells usually called primordial germ cells enlarge into follicles and degenerate by atresia, though Bühler ('06) refers to a period of growth shortly after birth, then a cessation and remaining dormant. Winiwarter and Sainmont ('08), in the cat, state that all germ cells formed in the ovary up to thirty-six days after birth completely disappear, degeneration occurring at all stages, and Firket ('20) believes that all the embryonic germ cells of the rat completely degenerate and disappear.

Thus there can be no doubt of the degeneration and disappearance of enormous numbers of ova in many mammals some time before sexual maturity, but there remains a question of considerable importance. Do *all* the germ cells which are present at birth or soon after degenerate and disappear? A positive and definite claim that such is the case is made by B. M. Allen ('04), Winiwarter and Sainmont ('08), Kingery ('17), Firket ('20), Arai ('20), and Butcher ('27) for the rabbit, pig, cat, mouse, and rat. Some others are not so definite as to the number which degenerate, stating that 'the vast majority,' 'almost all,' 'many' degenerate before sexual maturity—for example, Schulin ('81), Lange ('96), Hansemann ('12), Kingsbury ('14), Kirkham ('16), Brambell ('27), and Schotterer ('28) for the cat, dog, mouse, and man. Of these, all but Schotterer state their belief that some of these early ova may remain dormant for a long time and later become functional. In man Bühler ('06), Felix ('12), and Simkins ('28) seem to agree that most of those present at birth remain dormant until sexual maturity. There is so great difference here and so evenly balanced opinions that the question remains unsettled. Paladino ('87, '03) and Papanicolaou ('24) find definite evidence that the process of producing new ova, their growth or degeneration, and ovulation, is a continuous process persisting throughout the young and sexually mature period, Papanicolaou stating that no follicle can remain dormant, but must either grow or degenerate. Their conclusion would necessarily be that no embryonic germ cell could persist until sexual maturity. My own observations and conclusions would confirm and agree with those of Papanicolaou.

This uncertainty as to whether all or only a part of the early formed ova degenerate is not due to lack of material; while it is true that those who believe in a total disappearance have had more extensive material in both embryonic and postnatal stages, yet Kingsbury, Brambell, and others also have had ample material. It is rather due to the difficulty in distinguishing between germ cells of different generations. A primary follicle developed from a germ cell formed in a sixteen-day embryo does not differ in structure or form from one developed from a germ cell produced sixteen days after birth, or even sixteen days after sexual maturity; there is probably a continuous formation of germ cells, growth and degeneration of follicles, and different generations and stages overlap. It seems rather improbable that this question can be definitely proved one way or the other by direct observations, and the experimental evidence so far obtained is just as conflicting and uncertain. One must come to a conclusion on the basis of indirect evidence and logical induction; a careful consideration of the evidence leads me to the conclusion that probably all of the early formed germ cells degenerate and disappear some time before sexual maturity. In this I confirm and agree with B. M. Allen, Kingery, Firket, Arai, and Butcher.

3. Source of the definitive ova

Our question here is: What is the origin, or the source, of those cells which are the functional and definitive ova of the sexually mature animal? From the preceding section it seems highly probable that all the primitive ova of the embryo and young animal degenerate; but it is also clear that we cannot claim positively that no primitive ovum persists to become one of the definitive ova. However, it makes little difference whether one or several embryonic ova persist, the practical question is whether the great majority of ova functional in the adult are new formations produced during the postnatal period, or represent primitive ova persisting from their origin in the early ovary. Still further, if only a few ova produced in the young and adult animals can be proved to arise by transformation of germinal epithelial or peritoneal cells, this is sufficient to demonstrate the germinal potency and capacity of such tissue cells.

The fact that such large numbers of primitive germ cells disappear before maturity renders it probable that others arise to take their place; I am convinced that there can be but few, if any, of the germ cells present in the ovary of the newborn rat which do not degenerate and disappear. In addition, there is evidence that new germ cells arise in the ovary after birth. The germ cells in the ovaries of animals from birth to three days after are forming primitive follicles (figs. 18, 20), and very few naked oocytes can be discovered. In animals six days after birth we find masses of naked oocytes in or below the tunica albuginea and enlarged cells in the germinal epithelium (fig. 25); from nine to eleven days, these become primitive follicles and the germinal epithelium makes further contributions. For example, in figures 26 and 27 are seen some enlarging cells in the surface layer of the ovary and other cord-like proliferations which contain enlarged cells. From fourteen to sixteen days, still further masses of naked oocytes are found just beneath the surface of the ovary, while those formed at six days have become surrounded by follicle cells. By nineteen days after birth, the ovary is filled with graafian follicles, while primitive follicles and naked oocytes are rare and few enlarged cells can be found in surface layers. At twenty-three days, when maturation of ova and degeneration are near their maximum, oocytes and primitive follicles are at their minimum and the germinal epithelium seems to be little active in forming enlarged cells. Between twenty-six and twenty-nine days after birth, the oocytes and primitive follicles begin to increase in numbers and many large cells are present in the germinal epithelium, or cords are growing inward from it; finally, by thirty-six to thirty-nine days, these activities reach something of a maximum and are more profound than at any time since the embryonic period.

This series of observations is so consistent and clear that it seems definitely to establish the fact that the germinal epithelium produces large numbers of ova during the youth of the animal and especially a short time before the attainment of sexual maturity. In addition, there is distinct proof of the gradual transformation of germinal epithelial cells into follicles. One series of figures (27 to 31) represents the gradual enlargement of a surface cell in place, its enclosing by neighboring cells as follicle cells, and the movement of this into the tunica albuginea. Figures 34, 37, 39, and 38 are another series demonstrating the same thing, and 32, 33, 35, and 39 show the young follicles embedded in the albuginea. While the enlargement of a single germinal epithelial cell and its transformation into a follicle is the usual method of forming new ova, there is another method frequently found: the germinal epithelium proliferates into tube- or cord-like ingrowths which soon become separated from the surface; some of the cells in the cord enlarge to form ova and others surround them in a follicle (figs. 26, 32, 33, 35, 36), and the cord later breaks up into distinct follicles.

An analysis of the literature indicates that the formation of young ova from the germinal epithelium of the ovary during the period between birth and puberty is a rather common occurrence in mammals. It has been found in the cat, dog, mouse, rabbit, rat, guinea-pig, bat, and one of the lemurs, doubtless in others. This has been worked out in careful detail, especially in the mouse and rat. Harz ('83) was the first to carefully study and describe, in the young and adult, the enlargement of germinal epithelial cells, the growing of neighboring cells around them to form a follicle which then pushed into the ovary to form first a primitive and later a graafian follicle. Lange ('96) confirmed this and found it took place until late in the life of the mouse; ingrowing cords of cells also produced ova. Kingery ('17) even more carefully worked out the details and demonstrated the certainty of this method; the definitive ova originated only in this way, all earlier ova degenerating; he believed such new formation ceased at puberty. Recently, Brambell ('27) has confirmed Kingery's conclusions, though he thinks there is new formation in the adult. In the rat the evidence is even clearer and more positive. By an actual count of the ova present at various ages, Arai ('20) was convinced that all embryonic ova disappeared and definitive ova were postnatal in origin, and the new formation continued for at least a year after birth, that is, through the large part of the adult life. Butcher ('27) has again investigated the matter in the rat from birth to ninety days after; he finds the enlargement of germinal epithelial cells in place, their enclosing by adjacent cells to form follicles, and traced the ova in the follicles to their maturation. Since, in the adult, he followed the entire process of formation of new ova from the germinal epithelium to mature ova and found it a regular and common process, there can be no doubt of the facts. My own observations confirm those of Butcher completely, with the addition that some cord-like ingrowths from the surface of the ovary may also produce ova. Cowperthwaite ('25), who does not admit there is any new formation of ova, nevertheless finds precisely the same enlargement of surface cells and transformation into follicles. Edgar Allen ('23) describes a complete new formation of ova from the germinal epithelium of the adult at each oestrous period; however, he is in error in stating that mitosis must precede this and that enlargement does not occur at the point of origin.

Another important line of evidence is that of Papanicolaou ('24) in the guinea-pig; here there is found a continuous process of new formation of ova from the germinal epithelium from the time the gonad is differentiated until the cessation of sexual activity; even during pregnancy the same process goes on less actively. In young animals an excess of ova are produced, in older ones an excess of follicle or luteal cells, interstitial cells being merely groups of aborted oocytes. This demonstration that the same activity of the germinal epithelium may result in the formation of ova, follicle cells, luteal and interstitial cells explains the interpretation of certain authors like League and Hartman ('25) that only anovular follicles may be produced by germinal epithelial activity. Such interpretations seem clearly to be due to the failure to follow the process for a long time or through many stages. Paladino ('03), in the dolphin, finds a similar continuous formation of ova throughout the entire reproductive period.

Without going into details, it may be stated that similar activity of the germinal epithelium in producing new ova in young and adult animals has been described for the cat by Harz ('83), Coert ('98), and Winiwarter and Sainmont ('08); in the dog, by Bonnet ('91) and Felix ('12); in the rabbit, by Coert and B. M. Allen ('04); in the bat, by Van Beneden ('80), and in the lemur, by Rao ('28). The chief opposing conclusions are those of Kingsbury ('14) in the cat and Kirkham ('16) in mouse. The former found no evidence of any new formation later than about three weeks after birth, and the latter denied that ova could form at any time after birth. But the evidence of Harz, Lange, Kingery, and Brambell in the mouse shows that Kirkham is mistaken. Van Beek ('24), in the cow, found the activity of the germinal epithelium limited to a few months after birth. Schottländer ('93) and Regaud and Policard ('01) state that ingrowths of germinal epithelium occur in the adult dog ovary, but no ova are produced; this is opposed by Bonnet and Felix. Waldeyer ('70) and Schulin ('81) found enlarging ova in the germinal epithelium of young and adults of several mammals; they interpreted this to mean a retardation of purely embryonic processes for a long time; the more recent studies would compel a change in this interpretation. In regard to the human the evidence is far from clear; Nagel ('88, '89), Waldeyer ('03), Felix ('12), and Simkins ('28) all agree that no new ova are produced in the adult or even in the young after two or three years; Simkins says not after the embryo of the fifth month. On the contrary, Amann ('99) and Fellner ('09) found large cells or primordial ova in the germinal epithelium of adults, and believed these had recently originated there; there are other similar conclusions.

4. Meiosis and definitive ova

A certain definite and regular series of nuclear changes was considered by Winiwarter and Sainmont ('08) to be the test of a mammalian germ cell. These changes were the usual synaptic or meiotic changes, preceded by other chromatin changes just as necessary, though not so clearly marked. These authors believed there existed three proliferations of the germinal epithelium of the ovary, one entirely embryonic, the second begins about the time of birth and continues for about thirty-five to forty days, the third begins only about thirty-six days after birth. In all of these proliferations the meiotic changes occur, but all cells of the first two proliferations degenerate and only the third form the definitive ova;

enlarged cells in the germinal epithelium of the ovary at any period are only hypertrophied cells and do not form ova; meiosis does not take place in them. Three things of importance should be noted: 1) all ova are transformed germinal epithelial cells; 2) the definitive ova are all postnatal in origin; 3) meiosis occurs at least three times—in the embryo, shortly after birth, and some time before puberty. Large cells of the germinal epithelium of young and adult ovaries have been followed into the production of follicles by a number of authors, hence Winiwarter and Sainmont must be in error in their conclusion on this fact; but they are confirmed in the postnatal origin of definitive ova. Most workers seem to agree in finding meiosis abundant from late embryonic stages to a few days after birth and at no other time. If synapsis occurs as described by the above-mentioned authors in the late postnatal ova these must be different in character, or at least far less apparent, since they have rarely been noted by other workers; the only one recording this is Rao ('28), who states that in one of the lemurs the germinal epithelium of the ovary in pregnant animals proliferates cells which become ova and their nuclei pass through all the changes described by Winiwarter and Sainmont for the cat. All other investigators looking for this series of changes have not found it; one must therefore conclude that it is not of common occurrence in ova formed after birth.

Is meiosis a necessary phase in the cycle of the ovum in mammals? Kingery carefully tested this query in the mouse and concluded it was not; meiosis took place in all embryonic ova, but these all degenerated; the definitive ova arising postnatally show no evidence of such changes. Yet Kingery was able to trace the enlarged cells from the germinal epithelium as they entered the ovary and became transformed into follicles which later became mature graafian follicles of the adult; he was, therefore, warranted in concluding that the fate of these cells shows they are ova, and since they exhibited no synapsis this cannot be accepted as a test of their germ-cell nature. Cowperthwaite ('25), in the albino rat, takes as her thesis that meiosis is an absolutely necessary phase of germ-cell history. Like Kingery, she finds large cells in the germinal epithelium of the young ovary, these move into the ovary and become follicles similar in structure and appearance to all other follicles; but none of these cells arising in the postnatal ovary exhibit any synapsis. She concludes that the cells and follicles so produced cannot be considered as germ cells; however, since it is admitted that there is no distinguishable difference from follicles produced from these cells and those from embryonic ova, it is impossible to accept her conclusion and interpretation. Brambell ('27), in the mouse, found no synapsis later than four days after birth, but there are ova produced from the germinal epithelium of the ovary even in the adult, hence the meiotic phenomena cannot be necessary. Still again the question was tested by Butcher ('27) in the rat; with the conflicting preceding conclusions before him, careful attention was paid to this question. All definitive ova are found to arise postnatally and none of these show any of the meiotic changes, but they pass through the regular maturation changes as the ova of the adult animal; his conclusion is clear and positive:

> If a transition of peritoneal cells into germ cells can be demonstrated during pre-pubertal life, and then one is able to trace them through the first maturation division, there should be no doubt about their true oogenetic properties. By no means should the absence of a demonstrable synaptic stage be correlated with a loss of germinal potentiality.

Though I had completed my study of the ovary of the rat before Butcher's paper appeared, I reviewed my entire lot of sections of ovaries from a fourteen-day embryo into sexual maturity; I again concluded, as I had previously, that Kingery and Butcher were entirely correct, and I am glad to confirm their observations and conclusions.

With these facts before one: degeneration of embryonic ova; new formation of ova by a transformation of germinal epithelium in the young and adult ovary; ability to follow these cells into the development of a normal follicle; the formation of the polar body of the ova of such follicles in perfectly normal manner; the presence of synapsis and meiotic changes in embryonic ova; the absence of such changes in ova formed postnatally; one seems justified in making but one conclusion, namely, that meiosis is not a necessary phase of the history of the ovum.

It may be urged that so many of our biological generalizations are based on, and harmonious with, the hypothesis of synapsis of chromosomes in germ cells that the opposing conclusions so strongly substantiated in the mammals cannot be accepted. To this two replies may be suggested. First, our present hypothesis may be a false one; the hypothesis itself should be tested in the light of the opposing evidence, rather than the objections denied and discarded. Secondly, whatever is really significant and necessary in our concept of synapsis may take place in a different manner than we have believed; it is possible that there may be homologous chromomeres which pair in the stage of distributed chromatin, and not in the condensed chromosomal state. Metz ('16, '21, '22), in some of the Diptera, found there might be only a very rudimentary synapsis involving only a small portion of the chromosomes, in others no leptotene or synapsis could be found, though diplotene threads were observed. He further calls attention to the fact that the chromosomes seem to pair in somatic mitoses, and conditions similar to synapsis in appearance can be observed. Walker ('26) makes similar observations by comparing meiosis with somatic telophases in rat, mouse, rabbit, and guinea-pig. He states that the chromosomes in somatic telophases split longitudinally before the nucleus becomes vesicular and the appearance of these chromosomes is similar to synapsis in germ cells. Furthermore, he finds that leptotene, synaptene, synizesis, and diplotene phases of chromatin can be observed and followed in every telophase of somatic mitoses. Walker concludes that meiosis in germ cells has the same significance as similar appearances in somatic mitoses, and no other. These observations emphasize the question raised above—the meiotic phenomena need reexamination, not in one or two specific cases, but as a general and essential process in germ-cell history.

5. *Experimental evidence*

In adult male mice, guinea-pigs, and rats it has been found that heat or x-rays will cause a degeneration of all germ cells of the seminiferous tubules, leaving only the Sertoli cells. Moore ('24) forced the testes back into the body cavity, Stieve ('24) exposed animals in a hot chamber, and Oslund ('26) used x-ray treatment. In all these experiments a single row of Sertoli cells in the seminiferous tubules remained after the treatment; with the return of the animals to normal conditions, the investigators found that normal active spermatozoa were again produced. Hence male germ cells may be derived from any of the epithelial cells of the tubules.

Female animals have been placed in heated chambers by Stieve ('24) and exposed to x-rays by Brambell, Parkes, and Fielding ('27, '28), the latter treating mice at birth and at three weeks old. By these treatments all oocytes and young follicles are killed and degenerate, and more slowly the large follicles generally degenerate, thus the ovaries are left without germ cells. The germinal epithelium proliferates and forms ingrowing cords, there being two such proliferations according to Brambell, Parkes, and Fielding. The latter experimenters also exposed adult rats to x-rays, but found no proliferations of the germinal epithelium. In the proliferated cords produced after treatment some large cells like spermatogonia were seen by the experimenters, but they stated that no ova could be found. Explanted pieces of testes and ovaries were tested by Champy ('26), who found growth of the epithelium, but no differentiation; on the other hand, Fano and Garofolini ('27), using pieces of ovaries of nineteen-day embryo chicks, found growth in the cultures, and also some enlargement of cells and a grouping into masses or nests; degeneration finally overtook these.

Bagg and Papanicolaou ('24) irradiated the thyroid gland with radium and discovered that even in the adults there was an increase in oogenetic processes, including the formation of new ova from the germinal epithelium. They think anything which causes an increase of blood flow to the ovary results in increased formation of ova from the germinal epithelium. Several experiments on regeneration of ovarian tissues after double ovariotomy are recorded. Davenport ('25) found regeneration in mice even at some distance from the site of the excised ovaries; the regeneration came from the peritoneum. Hanson and Heys ('27) used the albino rat; in eight cases out of 105 regeneration occurred. By sectioning the excised ovaries, it was possible to determine that the entire organs had been removed without injury or break, hence no ovarian tissue could have remained behind. Parkes, Fielding, and Brambell ('27) followed the same precautions in their mice; out of 121 cases, eleven showed a recurrence of oestrus and in at least eight of these the ovaries had been completely and cleanly removed and new ovarian tissue regenerated from the peritoneum. Haterius ('28), also in mice, found five cases of regeneration out of ninety-six, but because the number regenerating was small he concludes there must have been some old ovarian tissue left behind, even though sections of the excised ovaries showed them complete without break or injury. The results of others would seem to indicate that Haterius had a true regeneration from the peritoneum. Brambell, Fielding, and Parkes ('28), in some cases of recurring oestrus after double ovariotomy, used x-ray treatment and later killed the animals for examination. Histological examination of the regeneration showed ovarian tissue with degenerating oocytes and follicles like the normal ovaries treated with x-rays. In these there had been the same double proliferation of germinal epithelium, but no cells they could call ova.

Experiments with explanted germinal tissue seem to tell us nothing definite. In the treatment of gonads with x-rays and heat there is a general death and degeneration of the existing germ cells, the germinal epithelium is not killed, but undergoes proliferations of its tissue into ingrowing cords. The most that can be said of these cords is that sometimes there were larger cells, but they resembled spermatogonia and not ova, in other cases there seemed nothing like enlarged cells; Brambell, Parkes, and Fielding did find definitely ova in one animal so treated; they offer no explanation of this. All four of the ovariotomical experiments got some regeneration in a small number of cases; this is admitted as regeneration from the peritoneum by three, but not accepted as such by the fourth. These results from the various sorts of experimental treatment of mammals are less certain and convincing than the many careful cytological studies; they do not furnish us with any facts not already discovered by microscopic study, nor are they extensive or consistent enough to cause any change in conclusions drawn from the cytological studies.

SUMMARY

The entire ovary of the rat, including its germ cells, is produced by a proliferation of the peritoneal epithelium; germ cells are not a special type of cell which migrate into the gonad. The germ cells of the embryonic ovary become larger, assemble into nests, and undergo mitotic divisions until about eighteen days after insemination. They pass through definite leptotene, synizesis, and pachytene stages between the periods of the eighteen-day embryo and three days after birth. The germ cells of the nests become surrounded by follicle cells, the nests break up, and by three days after birth the entire ovary is filled with distinct primitive follicles. All of these begin to grow at about the same time, and about sixteen days after birth the ovary is filled with large graafian follicles; by twenty-one days, maturation is in progress and many ova and follicles are degenerating. Maturation reaches it climax about twenty-six days and degeneration, between twenty-nine and thirty-two days; at this period there are very few large follicles remaining. Between thirty-six and forty-five days, there is another period of growth of germ cells into graafian follicles, their maturation and degeneration. Ovulation is first observed about forty-five days after birth. Another series of changes occurs during this youthful period of the animal: the great activity of the germinal epithelium in forming new germ cells during the embryonic stages decreases after birth and reaches a minimum about twenty-three days; from this time there is a marked increase in the formation of new ova which reaches a maximum between thirty-six and thirty-nine days, but they continue to form from the germinal epithelium up to sexual maturity and also in the adult ovary.

The degeneration of ova which is so profound during the period preceding sexual maturity seems to involve all the germ cells formed in the embryonic ovary and many of those formed shortly after birth. It seems probable that none of these first-formed ova remain in the adult ovary, though it cannot be directly proved that a few may not persist. However, most of the definitive ova of the adult ovary have been formed during the postnatal period by direct transformation of the cells of the peritoneal or germinal epithelial surface of the ovary. This process of transformation seems to be a continuous one, and no ovum or follicle can remain long in a dormant condition—it must either grow and mature or else degenerate. Not only the present observations, but many others are so consistent and clear that there can no longer be any doubt of the fact that definitive ova of the mammal are transformed cells of the germinal epithelium, produced long after birth and even in the adult animal. This transformation occurs chiefly by an enlargement of a single germinal epithelial cell in its original position, the formation of follicle cells by smaller neighboring epithelial cells, and the pushing of the whole into the body of the ovary; however, some cord-like ingrowths from the surface may also produce ova and follicles.

It has been claimed that synapsis and other meiotic changes represent absolutely necessary phenomena and only the cells in which such changes can be distinguished and demonstrated are to be considered as true germ cells. But the entire process of new formation of ova has been followed from the germinal epithelium through enlargement, follicle formation and growth, into normal maturation and ovulation; such cells are obviously normal ova, though they have exhibited no recognizable synapsis and meiosis. Therefore, this assumed criterion of germ cells cannot be accepted.

BIBLIOGRAPHY

ALLEN, BENNET M. 1904 The embryonic development of ovary and testis of the mammals. Am. Jour. Anat., vol. 3, pp. 89–154.

ALLEN, EDGAR 1923 Ovogenesis during sexual maturity. Am. Jour. Anat., vol. 31, pp. 439–482.

AMANN, J. A. 1899 Über Bildung von Ureiern und primärfollikelähnlicher Gebilde im senile Ovarium. Festschr. f. Kupfer, S. 717–730.

ARAI, HAYATO 1920 On the postnatal development of the ovary (albino rat) with especial reference to the number of ova. Am. Jour. Anat., vol. 27, pp. 405–462.

BAGG, H. J., AND PAPANICOLAOU, G. N. 1924 Reactions in the female reproductive system following irradiation in the thyroids. Science, vol. 59, p. 448.

BALFOUR, F. M. 1878 On the structure and development of the vertebrate ovary. Quart. Jour. Micro. Sci., vol. 18, N.S., pp. 383–438.

BONNET, R. 1891 Grundriss der Entwicklungsgeschichte der Haussäugetiere. Berlin.

BRAMBELL, F. W. R. 1927 The development and morphology of the gonads of the mouse. I. The morphogenesis of the indifferent gonad and of the ovary. Proc. Roy. Soc., B, vol. 101, pp. 391–408.

——— 1928 Ibid. III. The growth of the follicles. Ibid., vol. 103, pp. 258–271.

BRAMBELL, F. W. R., PARKES, A. S., AND FIELDING, UNA 1927 Changes in the ovary of the mouse following exposure to x-rays. Part I. Irradiation at three weeks old. Proc. Roy. Soc., B, vol. 101, pp. 29–56.

——— 1927 Ibid. Part II. Irradiation at or before birth. Ibid., pp. 95–114.

BRAMBELL, F. W. R., AND PARKES, A. S. 1927 Ibid. Part III. Irradiation of the non-parous adult. Ibid., pp. 316–328.

BÜHLER, A. 1894 Beiträge zur Kenntnis der Eibildung beim Kaninchen und die Markstränge des Eierstockes beim Fuchs und Menschen. Zeitschr. f. wiss. Zool., Bd. 58, S. 314–339.

——— 1906 See Felix, '06.

BUTCHER, EARL O. 1927 The origin of the definitive ova in the white rat (Mus norvegicus albinus). Anat. Rec., vol. 37, pp. 13–30.

CHAMPY, CH. 1926 Sur les cultures d'épithélium germanitif in vitro. Compt. Rend. Soc. Biol. Paris, T. 94, pp. 1082–1084.

COERT, H. J. 1898 Over de ontwikkeling en den bouw van den geslachtsklier bij de zoogdiern meer in hat bijzonder van den Eierstock. Akad. Proefschrift, Leiden. (Zool. Jahresb., 1898.)

COWPERTHWAITE, MARION HAZEL 1925 Observations of pre- and postpubertal oogenesis in the white rat, Mus norvegicus albinus. Am. Jour. Anat., vol. 36, pp. 69–90.

DAVENPORT, CHARLES B. 1925 Regeneration of ovaries in mice. Jour. Exp. Zoöl., vol. 42, pp. 1–12.

FANO, G., AND GAROFOLINI, L. 1927 Culture in vitro of germinal epithelium from the embryo chick. Jour. Physiol., vol. 63. (Physiol. Abstr., vol. 12, p. 412.)

FELIX, W. 1906 Germ glands, in: O. Hertwig, Handbuch d. vergl. u. exp. Entwicklungslehre d. Wirbeltiere. Jena.

———— 1912 Keibel and Mall: Manual of human embryology. Lippincott & Co., New York.

FELLNER, O. O. 1909 Zur Histologie des Ovariums in der Schwangerschaft. Arch. f. mikr. Anat., Bd. 73, S. 288–305.

FIRKET, JEAN 1920 On the origin of germ cells in higher vertebrates. Anat. Rec., vol. 18, pp. 309–316.

FOULIS, JAMES 1876 On the development of the ova and the structure of the ovary in man and other mammals. Quart. Jour. Micro. Sci., vol. 16, N.S., pp. 190–220.

FUSS, A. 1912 Über die Geschlechtszellen des Menschen und der Säugetiere. Arch. f. mikr. Anat., Bd. 81, Abth. II, S. 1–22.

HANSEMANN, D. VON 1913 Über den Kampf der Eier in den Ovarien. Arch. f. Entwmech., Bd. 35, S. 223–235.

HANSON, F. B., AND HEYS, F. 1927 On ovarian regeneration in the albino rat. Proc. Soc. Exp. Biol. and Med., vol. 25, pp. 183–184.

HARGITT, GEO. T. 1925 The formation of the sex glands and germ cells of mammals. I. The origin of the germ cells in the albino rat. Jour. Morph. and Physiol., vol. 40, pp. 517–557.

———— 1926 Ibid. II. The history of the male germ cells in the albino rat. Ibid., vol. 42, pp. 253–306.

HARZ, W. 1883 Beiträge zur Histologie des Ovariums der Säugetiere. Arch. f. mikr. Anat., Bd. 22, S. 374–407.

HATERIUS, H. O. 1928 An experimental study of ovarian regeneration in mice. Physiol. Zoöl., vol. 1, pp. 45–54.

KINGERY, H. M. 1917 Oogenesis in the white mouse. Jour. Morph., vol. 30, pp. 261–316.

KINGSBURY, B. F. 1913 The morphogenesis of the mammalian ovary: Felis domestica. Am. Jour. Anat., vol. 15, pp. 345–388.

KIRKHAM, W. B. 1916 The germ cell cycle in the mouse. Anat. Rec., vol. 10, pp. 217–219.

KULESCH, L. 1914 Der Netzapparat von Golgi in den Zellen des Eierstockes. Arch. f. mikr. Anat., Bd. 84, Abth. I, S. 142–150.

LANGE, J. 1896 Die Bildung der Eier und der Graaf'schen Follikel bei der Maus. Verhand. phys.-med. Gesell. Würzburg, N.F., Bd. 30, S. 56–76.

LEAGUE, BESSIE, AND HARTMAN, CARL G. 1925 Anovular graafian follicles in mammalian ovaries. Anat. Rec., vol. 30, pp. 1–14.

METZ, CHARLES W. 1916 Chromosome studies on Diptera. II. The paired association of chromosomes in the Diptera and its significance. Jour. Exp. Zoöl., vol. 21, pp. 213–280.

———— 1922 Chromosome studies on the Diptera. IV. Incomplete synapsis of chromosomes in Dasyllis grossa Fab. Biol. Bull., vol. 43, pp. 253–266.

METZ, CHARLES W., AND NONIDEZ, JOSÉ F. 1921 Spermatogenesis in the fly Asilus sericeus Say. Jour. Exp. Zoöl., vol. 32, pp. 165–186.

MEVES, F. 1907 Ueber Mitochondrien bzw. Chondrioconten in den Zellen junger Embryonen. Anat. Anz., Bd. 31, S. 399–407.

MOORE, CARL RICHARD 1924 Properties of the gonads as controllers of somatic and psychical characteristics. VI. Testicular reactions in experimental cryptorchidism. Am. Jour. Anat., vol. 34, pp. 269–316.

NAGEL, W. 1888 Das menschliche Ei. Arch. f. mikr. Anat., Bd. 31, S. 342–423.

———— 1889 Über das Vorkommen von Primordialeiern ausserhalb der Keimdrüsenanlage beim Menschen. Anat. Anz., Bd. 4, S. 496–498.

OSLUND, ROBERT M. 1926 Cryptorchid testes and testicular hormone production. Amer. Jour. Physiol., vol. 77, pp. 76–82.

PALADINO, G. 1887 Ulteriori ricerche sulla distinzione e rinnovamento continuo del parenchima ovarico dei mammiferi. Anat. Anz., Bd. 2, S. 835–842.

———— 1903 Sulla rigenerazione del parenchima ovarico e sul tipo di struttura dell 'ovaja di Delfina. Rend. Acad. Sci. Fis. e mat. Napoli, 42, pp. 289–298.

PAPANICOLAOU, GEORGE N. 1924 Ovogenesis during sexual maturity as elucidated by experimental methods. Proc. Soc. Exp. Biol. and Med., vol. 21, pp. 393–396.

PARKES, A. S., FIELDING, UNA, AND BRAMBELL, F. W. R. 1927 Ovarian regeneration in the mouse after complete double ovariotomy. Proc. Roy. Soc., B, vol. 101, pp. 328–353.

PRATT, B. H., AND LONG, J. A. 1917 The period of synapsis in the egg of the white rat, Mus norvegicus albinus. Jour. Morph., vol. 29, pp. 441–460.

RAÔ, C. R. NARAYAN 1927 On the structure of the ovary and the ovarian ova of Lorica Lydekkerianus, Cabr. Quart. Jour. Micro. Sci., vol. 71, pp. 57–74.

RAUH, WALTER 1926 Ursprung der weiblichen Keimzellen und die chromatischen Vorgänge bis zur Entwicklung des Synapsisstadiums. Beobachtet an der Ratte (Mus decum. alb.). Zeitschr. f. Anat. u. Entwgsch., Bd. 78, S. 637–668.

REGAUD, CL., ET POLICARD, A. 1901 Fonction glandulaire de l'épithélium ovarique et de ses diverticules tubuliformes chez la chienne. Compt. Rend. Soc. Biol. Paris, T. 53, p. 615.

RUBASCHKIN, W. 1909 Ueber die Urgeschlechtszellen bei Säugetiere. Anat. Heft., Bd. 39, S. 603–652.

———— 1910 Chondriosomen und Differenzierungsprozesse bei Säugetier Embryonen. Anat. Heft., Bd. 41, S. 399–432.

———— 1912 Zur Lehre von der Keimbahn bei Säugetieren. Anat. Heft., Bd. 46, S. 345–412.

SCHOTTERER, ANTON 1928 Beitrag zur Feststellung der Eianzahl in verschiedenen Altersperioden bei den Hünden. Anat. Anz., Bd. 65, S. 177–192.

SCHOTTLÄNDER, J. 1893 Über den Graaf'schen Follikel, seine Entstehung beim Menschen und seine Schicksale bei Mensch und Säugetieren. Arch. f. mikr. Anat., Bd. 41, S. 219–294.

SCHULIN, KARL 1881 Zur Morphologie des Ovariums. Arch. f. mikr. Anat., Bd. 19, S. 442–512.

SIMKINS, CLEVELAND S. 1928 Origin of the sex cells in man. Am. Jour. Anat., vol. 41, pp. 249–294.

SKROBANSKY, K. VON 1903 Beiträge zur Kenntnis der Oogenese bei Säugetieren. Arch. f. mikr. Anat., Bd. 62, S. 607–668.

STIEVE, H. 1924 Kastration durch Hitze mit nachfolgender Wucherung des Keimepithels. Zeitschr. f. mikr.-anat. Forsch., Bd. 1, S. 200–207.

TSCHASCHIN, S. 1910 Über die Chondriosomen der Urgeschlechtszellen bei Vögelembryonen. Anat. Anz., Bd. 37, S. 597–607, 621–631.

VAN BEEK, W. F. 1924 Die Entwicklung des Eierstockes beim Rinde. Zeitschr. f. Anat. u. Entwgsch., Bd. 71, S. 458–558.

VAN BENEDEN, ED. 1880 Contribution à la connaissance de l'ovaire des mammifères. Arch. de Biol., T. 1, pp. 475–550.

WALDEYER, W. 1870 Eierstock und Ei. Leipzig.

——— 1903 Die Geschlechtszellen, in: O. Hertwig, Handbuch vergl. u. exp. Entwicklungslehre d. Wirbeltiere. Jena.

WALKER, C. E. 1926 The meiotic phase in certain mammals. Proc. Roy. Soc., B, vol. 99, pp. 366–374.

WINIWARTER, H. VON, ET SAINMONT, G. 1908 Nouvelles recherches sur l'ovogénèse et l'organogénèse de l'ovaire des mammifères (chat). Arch. de Biol., T. 24.

EXPLANATION OF PLATES

All figures were drawn with the aid of the camera lucida; not only the outline of the cells and nuclei, but the form and size of the nuclear contents have been so made. In reproduction the figures have been reduced to three-fourths the original size; the magnifications refer to the reduced figures.

PLATE 1

EXPLANATION OF FIGURES

All figures × 1016

1 Fourteen-day embryo. A little over one-half the thickness of the ovary is represented. Note the large cell in the surface epithelium and others deeper.

2 Fifteen-day embryo. Edge of the ovary; in the surface epithelium are large cells and mitoses, similar things show in deeper layers. Some stroma cells forming.

3 Sixteen-day embryo. Edge of the ovary with some of the germinal epithelial cells slightly larger; the deeper cells are considerably larger and tend to group into masses.

4 Embryo of sixteen days eighteen hours. The edge of the ovary, to the right. Large germ cells are forming nests; fibrous tissue is forming about the nests, but no tunica albuginea has yet formed.

5 Embryo of sixteen days eighteen hours. Portion of another ovary showing the deeper portion. In one nest are degenerating cells, with others in mitosis. The nuclei are spherical in form, but vary in size.

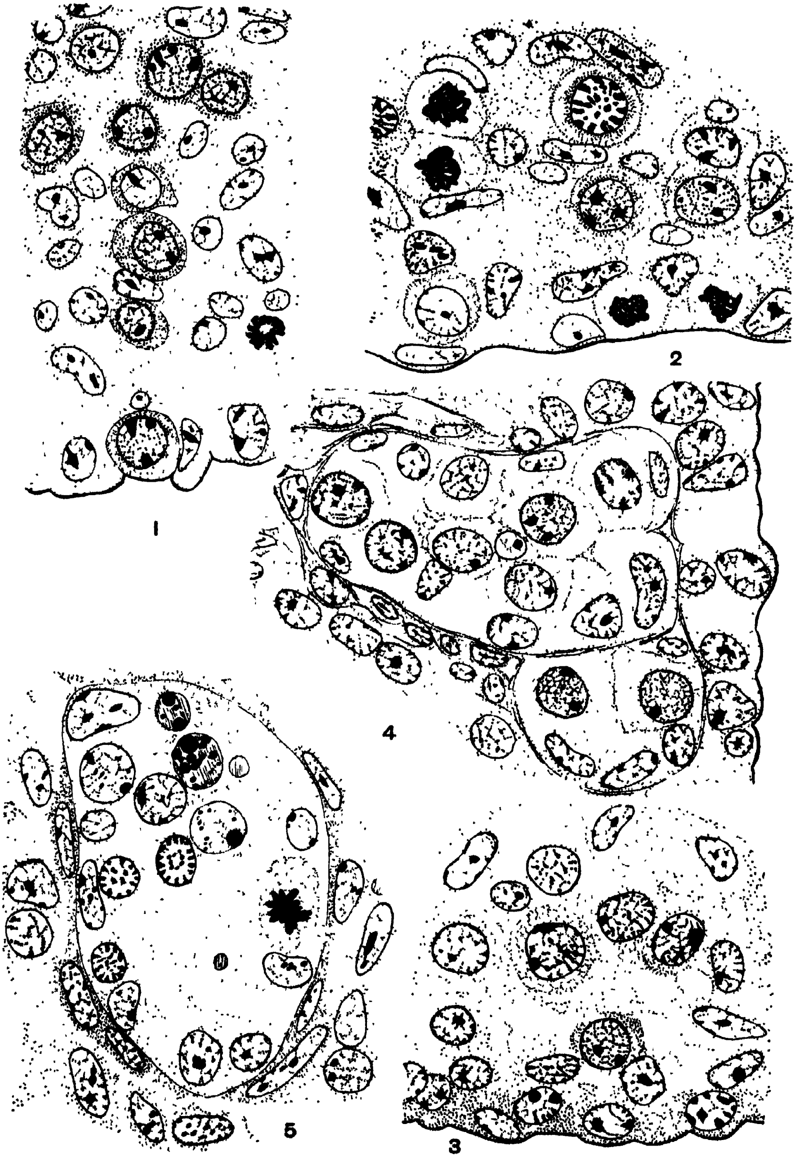
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PLATE 2

EXPLANATION OF FIGURES

Figures 6, 7, 11, 12, 15, × 1016; figures 8, 9, 10, 13, 14, × 1847

6 Embryo of sixteen days eighteen hours, dorsal portion of the same ovary as figure 5. In this region a tunica albuginea is forming between the germinal epithelium and the egg nests. Parts of two nests shown, in one mitosis abundant.

7 Embryo of seventeen days twenty hours. The edge of the ovary is represented, showing the germinal epithelium with two large cells just leaving and working back into the ovary. In the large nuclei of the cell nest synizesis is about to begin.

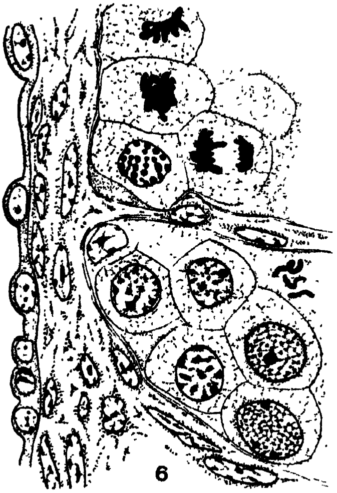
8 to 10 Embryo of seventeen days twenty hours. Nuclei of large cells from the egg nests of the same ovary as figures 7 and 11. In figures 8 and 9 the leptotene condition is shown; in figure 10 synizesis is starting.

11 Embryo of seventeen days twenty hours. Same ovary as figures 7 to 10. Portion of an egg nest from the basal or dorsal region of the ovary. Some nuclei show mitosis, others are degenerating, still others in synizesis.

12 Embryo of eighteen days sixteen hours. Egg nest from near the edge of the ovary. All the large nuclei are in synizesis and two nuclei in late stages of degeneration.

13, 14 Embryo of eighteen days sixteen hours. Same ovary as figure 12. Figure 13, a nucleus in early synizesis; figure 14, stage of dense contraction.

15 Embryo of nineteen days fourteen hours. Nest of germ cells sharply limited by connective-tissue stroma. No follicles are yet present. The nuclei are in synizesis, contraction, or degeneration.



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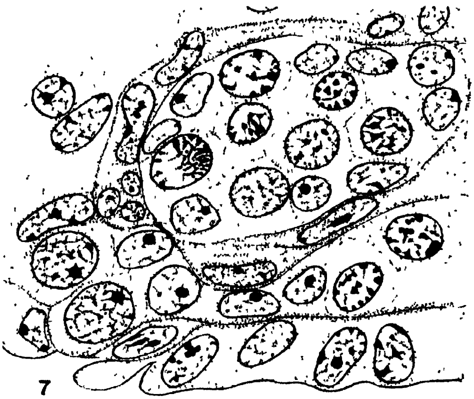

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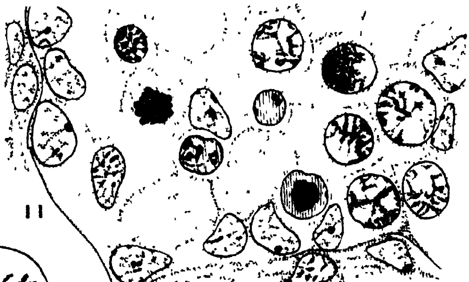

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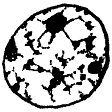

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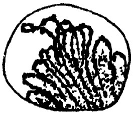

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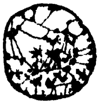

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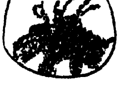

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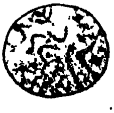

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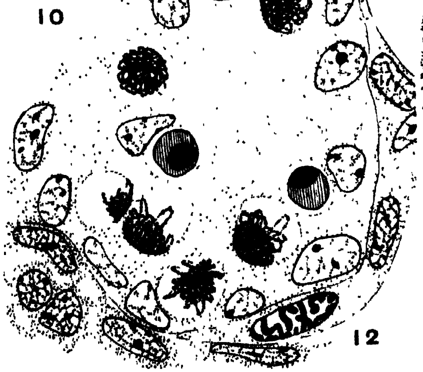

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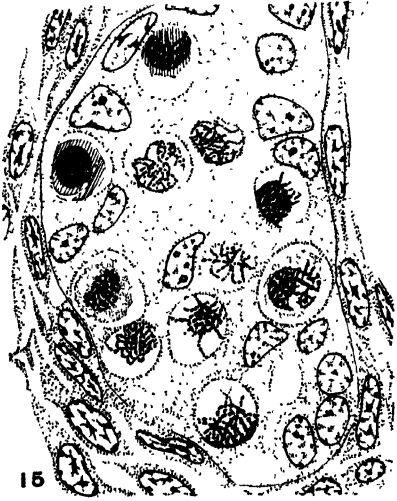

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PLATE 3

EXPLANATION OF FIGURES

Figures 16, 18, 20, 21, 24, × 1016; 17, 19, 22, 23, × 1847

16 Embryo of twenty days twelve hours. Edge of the ovary, with one germ cell still in the germinal epithelium; immediately below is another germ cell separated from the surface by a delicate tunica albuginea. Follicle cells are surrounding the germ cells; nuclei of germ cells in pachytene stage.

17 Embryo of twenty days twelve hours. Nucleus of an oocyte from the same ovary as figure 16, the chromatin showing the pachytene condition.

18 Newborn animal. Egg nests from near the edge of the ovary. Some germ cells have developed into primitive follicles and others are forming; stroma abundant. Chromatin of nuclei still in pachytene stage or forming the resting condition; two degenerating germ cells present.

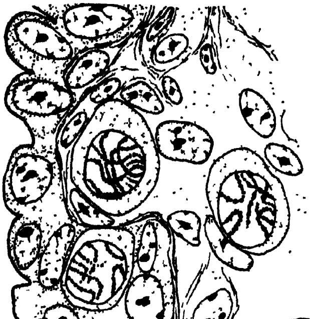
19 Newborn animal. Nucleus from a germ cell of the same ovary as figure 18, showing the details of chromatin arrangement.

20 Three days after birth. Edge of ovary. In this section of the ovary no albuginea is present, but stroma tissue is shown. One primary follicle is shown and others are forming; some cells of the nest have recently come from the germinal epithelium.

21 Three days after birth. Edge of the same ovary as figures 20, 22, 23. In the germinal epithelium is a much enlarged cell with the chromatin in pachytene condition; similar cells are present in deeper nests. Stroma is becoming abundant.

22, 23 Three days after birth. Nuclei of germ cells of the same ovary as figures 20, 21; in figure 22 the chromatin in loops, figure 23 shows the resting condition.

24 Six days after birth. Surface of ovary and tissues below, a definite but delicate tunica albuginea present; groups of germ cells just below the albuginea; no follicles present.



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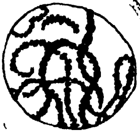

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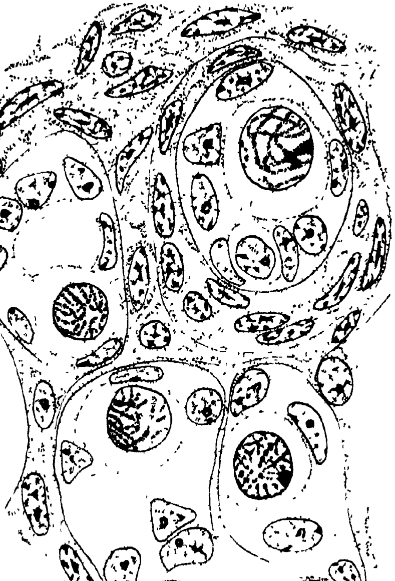

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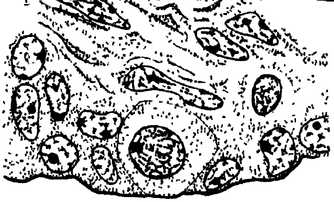

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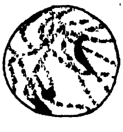

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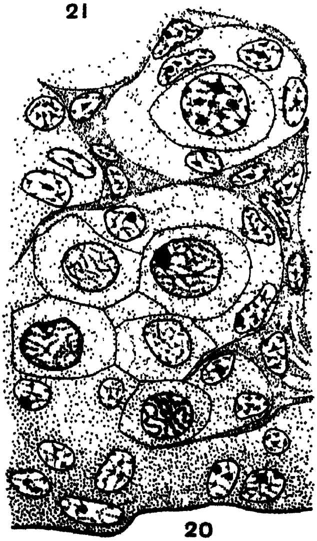

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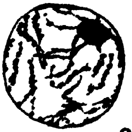

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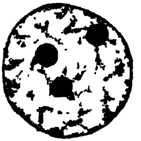

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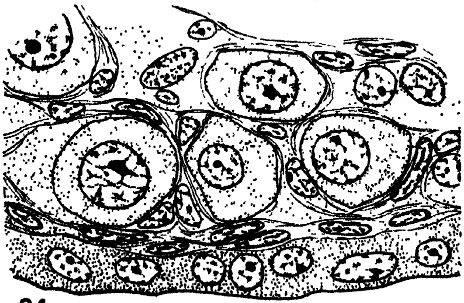

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PLATE 4

EXPLANATION OF FIGURES

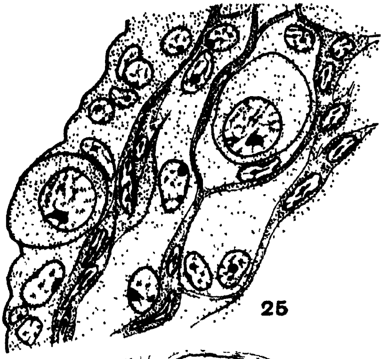
All figures × 1016

25 Six days after birth. Surface of the same ovary as figure 24. In the germinal epithelium a large germ cell and another beneath, similar to the germ cells in nests deeper in the ovary.

26 Nine days after birth. Edge of ovary. The stroma and tunica albuginea are much thicker; the germinal epithelium has formed a solid cord extending into the albuginea, the cord being partly divided into two groups with some nuclei considerably larger. Similar masses of cells a little deeper in the ovary contain enlarged germ cells, these deeper cords being epithelial ingrowths. Similar conditions are found in ovaries of sixteen, twenty-nine, thirty-six, and forty-five days after birth.

27 Eleven days after birth. Edge of ovary with well-marked albuginea. One of the germinal epithelial cells is enlarged and has a spherical nucleus, this will form an ovum later; just beneath the germinal epithelium is one of the surface cells forming an oocyte. In the albuginea are two primary follicles.

28 to 31 Nineteen days after birth. Portions of the surface of the same ovary, showing different stages in the enlargement of a surface cell and its development into a follicle. In figure 28 surface cell enlarged; figure 29, smaller cells surrounding the enlarged cell; figure 30, the large cell completely surrounded by small cells and being pushed deeper; figure 31, a primary follicle has developed and this is being pushed into the albuginea.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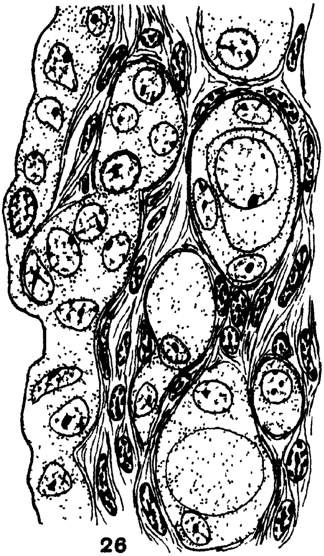
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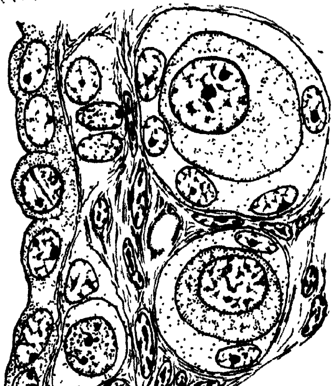
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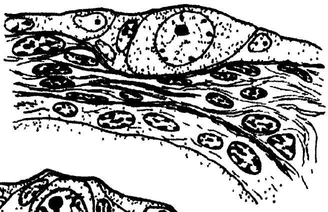
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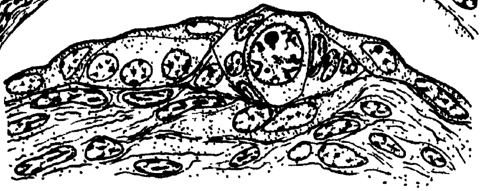
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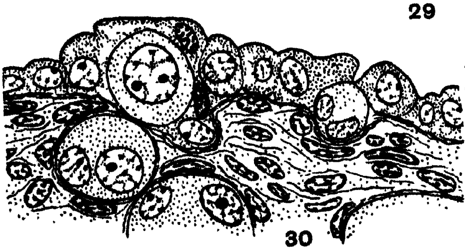
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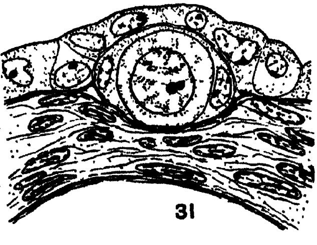
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PLATE 5

EXPLANATION OF FIGURES

All figures × 1016

32 Twenty-nine days after birth. Germinal epithelium thickened into a nest of cells; one of these is greatly enlarged and is forming a germ cell. In the albuginea is part of a group of epithelial cells formed in the same manner.

33 Twenty-nine days after birth. Some ovary as figures 32 and 34. The germinal epithelium is thin and peritoneal-like, the albuginea thick; in the latter is part of a solid cord of cells parallel to the surface and formed by a surface ingrowth. Within this cord are two large germ cells, one cut through the center and the other to one side of the nucleus.

34 Twenty-nine days after birth. Same ovary as figures 32 and 33. In the germinal epithelium one cell is slightly enlarged; below this other cells in the albuginea have formed distinct oocytes and are being surrounded by follicle cells; probably these cells originated in the surface layer.

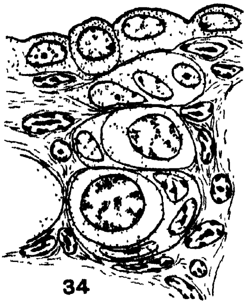
35 Thirty-six days after birth. The germinal epithelium has thickened into two pockets and part of a similar cord is surrounded by albuginea. From such groups germ cells may develop.

36 Thirty-six days after birth. Another portion of the same ovary as figures 35, 37, and 38. Several germinal epithelial cells are beginning to enlarge. In the albuginea is part of a large cord of cells, of epithelial origin, in which two definite oocytes have formed.

37 Thirty-six days after birth. Same ovary as figures 35, 36, and 38. A single enlarged germinal epithelial cell which has been transformed into a germ cell; it is being overgrown by the smaller epithelial cells to form a follicle.

38 Thirty-six days after birth. Same ovary as figures 35 to 37. Such a developing germ cell in the surface layer of figure 37 may form a complete follicle while it is still in the surface; such a condition is represented in the present figure, 38. It seems more common to find that the follicle is not completed until the cell is surrounded by the albuginea.

39 Forty-five days after birth. Ovulation has occurred in this ovary and large corpora lutea are present. The germinal epithelium is still active in forming germ cells, one of which is here shown, enlarged and pushing into the albuginea.



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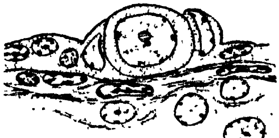

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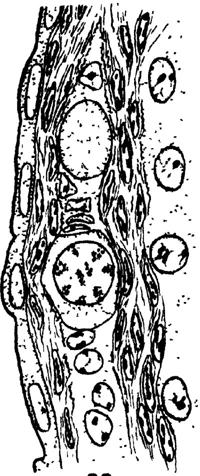

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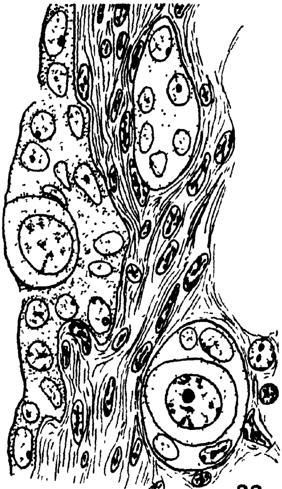

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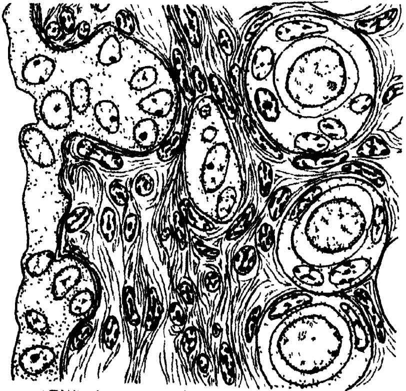

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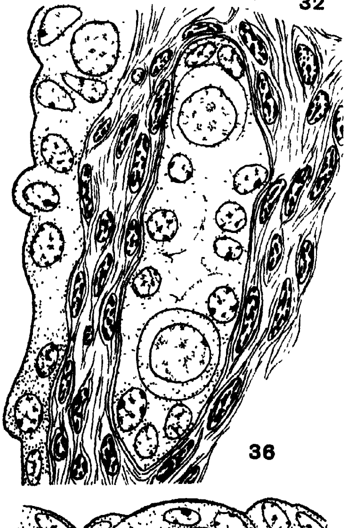

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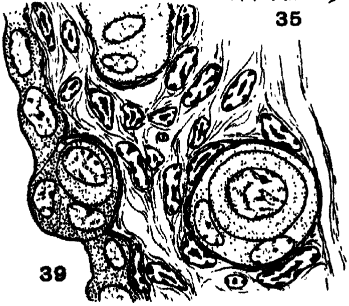

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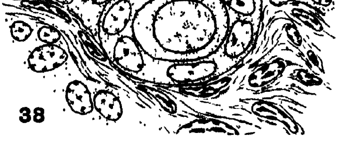

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THE FORMATION OF THE SEX GLANDS AND GERM CELLS OF MAMMALS

IV. CONTINUOUS ORIGIN AND DEGENERATION OF GERM CELLS IN THE FEMALE ALBINO RAT

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TWO PLATES (EIGHT FIGURES)

AUTHOR'S ABSTRACT

The current view in mammals is that ova are formed in the embryo in large numbers and stored in the ovary as a reserve to be drawn on through adult life, they may persist for long periods in a latent condition, a few resuming active development at each recurring oestrous period; new ova are not and cannot be added.

An alternative hypothesis has been presented by several authors: New ova are formed throughout life, from the embryonic period through the time of sexual maturity; each ovum so produced must at once begin its growth and development or die; long latency of oocytes and primary follicles is not possible and does not occur. There is a constant degeneration of most follicles, and only a few come to ovulation. A study of the albino rat presents evidence which is consistent with the second view and supports it more strongly than it does the older hypothesis. Similar evidence is found in other mammals. It seems probable that the newer hypothesis is the correct one, at least in some mammals, further investigation may show it to be of general application.

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INTRODUCTION

Among invertebrates the germ cells develop in the same manner in both sexes, and this is true in lower vertebrates. Whether there be a single period in which germ cells are perfected or whether this is a periodic and seasonal phenomenon, almost all eggs and sperm are developed and ripened at the same rate and discharged either at once or within a very brief interval. In the males of higher vertebrates there is no great difference from this condition, sperm formation is often a continuous process throughout the year and during the whole life; furthermore, when spermatocytes are formed, their development immediately follows—resting or latent periods do not occur. In females it has generally been assumed that a different state of affairs exists. The point of view is well represented by the statement of Pearl and Schoppe ('21): "It has been held as a basic biological doctrine that during the life of the individual there neither is nor can be any increase in the number of primary oocytes beyond those laid down when the ovary is formed." This is largely an assumption and, at least until recently, has not been subjected to adequate test and investigation. It is true that Pearl and Schoppe did not confirm this in birds, and cases have been cited in mammals which are not in harmony with the assumption, but they have been incidental references arising in the course of other investigations.

Pflüger ('63) was probably the first to describe the ingrowths of tubes or cords from the germinal epithelium of mammalian ovaries to form 'egg tubes' out of which the ova were developed. In the cat he thought there was a periodic new formation of ova in adult ovaries by this means, which he compared with the periodic growth of plants. Paladino ('87) claimed there was a continuous destruction of follicles with a constant new formation of egg tubes and ova, the process going on from birth to old age in mammals. The intensity of the process varied with age, the maximum occurring during sexual maturity, though also taking place before and after this time. This was reaffirmed ('03) after a new study of the dolphin. Lange ('96), in the mouse, reported that about twenty-eight days after birth all of the ova and follicles in the ovary began to grow at the same time, and the great majority degenerated; between forty-two and fifty-six days a new proliferation of germinal epithelium began and from this new growth all the definitive ova were developed.

Studying the cat ovary, von Winiwarter and Sainmont ('08) described three proliferations from the germinal epithelium, the second corresponding to Pflüger's 'egg tubes.' The ova of two series had completely degenerated by thirty-six days after birth; at about three months the third and last proliferation was initiated, and from this all the functional ova of the adult were derived. No clear and detailed description was given of the time and method of degeneration of primary and secondary ova. Firket ('20) found two generations of germ cells in the rat, the first having completely disappeared by fifteen days after birth; he referred chiefly to the male, but implied that a similar degeneration and new formation took place in the ovary. By actual count of ova in rat ovaries of various ages, Arai ('20) was led to decide that all primary ova of the embryonic and early postnatal periods degenerated and were replaced by ova newly formed from germinal epithelial cells. There was a very rapid growth and degeneration of ova between four and twenty-three days after birth, and a slower degeneration and absorption up to sixty-three days.

Edgar Allen ('23) revived in part the old idea of Pflüger that there was a periodic formation of new ova. At every oestrous period during the adult life of mammals large numbers of new ova developed from the germinal epithelium; as many as 400 to 500 ova differentiated in a single ovary and all but about 1 per cent of these degenerated before the next oestrous cycle began. Allen, Kountz, and Francis ('25) added that the degeneration took place in late stages of follicular growth, in this case the ratio of degeneration amounting to 85 per cent. Thus it appears that immediately after formation the ova began their growth, rapidly developed into large graafian follicles, and degenerated before the onset of the next cycle with its own new formation and degeneration.

We have here introduced, at least by implication, the thought that once an oocyte is produced it must immediately proceed with its growth and development. This view is critically examined, discussed, and extended by Papanicolaou

('24) from studies on the guinea-pig. He found it possible to stimulate the origin of new ova by experimental means and also to accelerate the growth phenomena, this enabling him to more clearly follow the germ-cell cycle. He definitely concluded that oogenesis was a continuous and not a cyclic process, extending over the period from embryonic life to the end of sexual maturity. New ova are constantly being produced, even during pregnancy, and when once formed every primary follicle must grow or die; a long persistence in a latent condition is impossible for follicles of any size whatever, nor can any oocyte be reserved or held back for future development. The vast majority of newly formed oocytes degenerate at this stage or as young follicles, others as growing or mature graafian follicles, and only a small number of the latter come to ovulation.

The rat has lately been restudied by Butcher ('27), who confirms many of the results of Papanicolaou. He finds that many ova begin their growth at the same time, six or seven days after birth, and an extensive degeneration takes place between thirteen and twenty-four days, but degeneration may occur at any or all stages of growth. Along with this degeneration there is a continuous proliferation of the germinal epithelium and the production of new ova therefrom until about sixty-five days, when it decreases, but does not end until much later. Butcher expresses no opinion on the issue of possible latency of oocytes raised by Papanicolaou. In a recent paper (Hargitt, '30) I have confirmed many of the above results and briefly discussed the general problem.

Therefore, a hypothesis alternative and diametrically opposed to the current view is specifically presented. The current opinion is that already quoted above: All ova are formed in the embryo and thereafter no new ones are or can be produced; the majority of these remain quiescent and latent for months or even many years and a few resume their activity at each rhythmically recurring reproductive cycle during the entire period of sexual activity. The alternative hypothesis is, briefly: Ova are being newly formed from the germinal epithelium throughout the entire life from the embryo to old age, either continuously or cyclically; an oocyte once formed must begin its growth and development at once, or else it must die; it cannot remain in reserve and at rest for a long period to resume its development at some future time.

The issue is important and has not received the attention it deserves; it should be carefully investigated and critically examined. In what follows I present the results of a study of this problem; some facts and impressions have accumulated during the study of other phases of the germ cells, but they have been subjected to a careful consideration under this new viewpoint and the material itself reexamined.

OBSERVATIONS

There are two lines of observation or investigation to be considered: 1) The growth, development, and degeneration of ova in the ovary, especially evidence touching upon widespread or general waves of development and degeneration; 2) evidence regarding the new origin of ova and the relation of this to growth, development, and degeneration. A summary will be given of actual conditions observed in the ovary at different ages, which may be found in greater detail in another paper (Hargitt, '30).

1. Growth and degeneration of ova

In embryos of eighteen and nineteen days the germ cells are assembled into definite, radially arranged nests, which completely fill the ovary. The nuclei of germ cells are all in leptotene or synizesis stages and all are about in the same phase—not only the germ cells in nests, but others located in the surface layer of the ovary. During the nineteenth day some of these germ cells are degenerating, both before and after entering the synizesis stage. On the twentieth and twenty-first days, the ovary is even more tightly packed with egg nests, the nuclei of which are emerging from the densely contracted synizesis stage and present chromatin strands of the pachytene type. In figure 1 is a diagrammatic drawing which represents the closely packed egg nests, the individual ova not being shown. Degeneration of the ova in nests is still common. No great change is encountered in the ovary a few hours after birth (fig. 2), except for the completion of the ovarian capsule. The chromatin strands have lost their sharp outline and are beginning to assume a net-like condition characteristic of the 'resting' nucleus.

The egg nests have broken up by three days after birth and the ovary is filled with individual oocytes (fig. 3) most of which are surrounded by a single layer of follicle cells forming primary follicles. The nuclei of the oocytes contain a typical chromatin network, though a few in the tunica albuginea or germinal epithelium still present some phases of meiosis. All meiosis has been completed six days after birth and thereafter does not appear; at the same time practically all ova have become surrounded by a follicle of several layers. During the three- and six-day period, degeneration of oocytes seems to have come to an end. From this period to eleven days after birth, the follicles all begin to grow and we find the first evidence of follicular cavities; degeneration of oocytes either does not occur or is rare.

Until the close of about sixteen days, the chief change noticed is the continued growth of the follicles, all of which come to be vesicular; they completely fill the ovary, even pushing close against the germinal epithelium. Figure 4 is a drawing of an ovary from an animal fourteen days after birth; the increased number of closely packed follicles is apparent at a glance, but the figure does not represent the follicular cavities which are almost universally present. Between this time and twenty-one days, the follicles increase in size to become practically mature graafian follicles which crowd against the surface, while smaller ones fill the center of the ovary. Again the majority of the follicles are about the same stage of growth, with a very few scattered primary follicles. The nuclei of the ova in the graafian follicles are in the germinal-vesicle stage. From the sixth to the twenty-first day, degeneration of oocytes and follicles has not been noted.

Figure 5 is a drawing of an ovary from an animal twenty-three days after birth, and it represents at a glance the profound changes which are initiated about this stage; the size relations are even more marked, since figures 1 to 3 are drawn at about twice the magnification of the other figures. This ovary is filled with many graafian follicles which have attained their full growth and most others are nearing this condition; a very few small follicles are scattered through the ovary. Most of the ova within the large follicles either have completed the formation of the first polar body or possess a maturation spindle, others still have a germinal vesicle. In the center of the ovary are a great many follicles which contain fragmenting ova and others whose ova have been completely resorbed. This period, therefore, marks three great changes: 1) a completion of the growth of many ova; 2) the occurrence of typical maturation changes; 3) the beginning of a profound degeneration.

These three processes continue through the twenty-nine-day stage and become more pronounced. More and more of the follicles complete their growth, pass through maturation, their ova fragment, and degeneration follows. Figure 6, from a twenty-nine-day ovary, very clearly shows this; practically all the follicles are large graafian follicles in maturation, with remnants of degenerate follicles filling the center of the ovary. By thirty-two days after birth, only a small number of graafian follicles remain; there are almost no oocytes or primary follicles, and the center of the ovary is completely filled with small concentric masses of degenerate follicles, many others having entirely disappeared. Between thirty-two and thirty-six days, a large number of graafian follicles are found in ovaries, some with germinal vesicles in their ova and others with maturation spindles or polar bodies present; at the same time there is an increase in the number of large follicles with fragmenting and degenerating ova and an almost complete disappearance of the remnants of degenerate follicles in the center of the ovary. It appears likely that one generation of ova has completed its growth and degeneration and a second generation has arrived at a similar stage. By thirty-nine days, once more there are but few large graafian follicles ready for maturation, few small and growing follicles, but a great increase in the number of degenerating follicles. Figure 7 is a section of a thirty-nine-day ovary which displays these phenomena.

At about forty-five days after birth, for the first time, ovulation has taken place and corpora lutea are present (fig. 8). The ovary, in most respects, resembles those of adult animals, except that there are larger numbers of graafian follicles most of which are maturing or degenerating, and far more evidence of past degeneration. In a single ovary of this stage a fairly accurate count gave the following: seven corpora lutea, thirty-nine graafian follicles apparently ready for discharge, over fifty follicles in early degeneration, and a great many remnants of degenerating follicles.

2. Origin of new ova

During a portion of the embryonic period the large germ cells are increasing by mitotic division; in later embryos this does not occur, but cells of the surface constantly enlarge and push into the body of the ovary. These large cells are produced individually or in small groups, and not as distinct cords. No interruption of this process occurs at birth, but the same increase of germ cells continues through the period of three days after birth. Between three and six days, there is an increase in the number of large germ cells originating in the germinal epithelium and a large number of naked oocytes are found just within the surface of the ovary, while those present earlier have developed into primary follicles. The newly formed oocytes become primary follicles by eleven days after birth and there remain very few naked oocytes in any part of the ovary.

From eleven to sixteen days, there is again an increase in the accumulations of naked oocytes and primary follicles, and some isolated cells enlarge within the germinal epithelium. This process markedly decreases for a time, and by twenty-three days there are the smallest numbers of oocytes and primary follicles found at any period of development, whether embryonic, postnatal, or adult. Following this there are increases, both in naked oocytes, primary follicles, and especially in enlarged cells within the germinal epithelial layer. This culminates at thirty-six days after birth, when this condition is the most marked at any time since birth and there are more enlarged epithelial cells in the surface layer than there have ever been up to this time. Such a condition continues through thirty-nine days and is still apparent, though not so prominent, at forty-five days, when the first normal ovulation occurs; indeed, the process continues on into adult life.

DISCUSSION

From the observations recorded there may be noted several lines of evidence consistent and harmonious throughout. 1) The great majority of the oocytes present shortly after birth begin an apparently normal growth which starts at the same time in all. At twenty-three days, some graafian follicles have completed their growth and entered upon maturation changes. Between this and twenty-nine days, practically all ova become mature graafian follicles and more and more ova form maturation spindles and polar bodies, until finally, at about thirty-two days, only a few follicles remain and these are forming polar bodies. 2) During the same time there is a progressive increase in the number of degenerating ova and follicles, so that the center of the ovary becomes a mass of remnants of follicles with fragmenting ova, all of which are finally absorbed. At thirty-six days, there are left only a few concentric masses of follicular tissue scattered through the center of the ovary, which represent the great number of ova present at birth. Many ova have also degenerated in the condition of primary follicles. 3) The number of oocytes and primary follicles in ovaries and the activity of the germinal epithelium in forming ova decrease through fourteen, sixteen, and twenty-one days and reach the lowest ebb at twenty-three days. In ovaries of twenty-six and twenty-nine days there are increased numbers of oocytes and follicles and many large cells are produced from the germinal epithelium and move into the ovary. While not so constant and steady as the growth and degeneration, there is a continued increase, and in thirty-six to thirty-nine days the germinal epithelium is more active in forming large cells than at any time since the embryonic period. There is some decrease about the time of ovulation, but the new formation of ova continues into the adult period. 4) With the increase in number of oocytes from twenty-six to thirty-six days there is a renewal of the growth, maturation, and degeneration of ova, similar to the earlier cycle of the same changes.

If the cycle of changes outlined in the above four categories were independent of each other; if each one were completed before another began, it would be quite easy to demonstrate the precise significance of these processes. But the facts are quite otherwise: one phase overlaps another in point of time; various phases of one process, and also different processes, are occurring at the same time; one ovary may contain every stage of development, of degeneration, and of new formation of ova, and all of these will be discernible to one studying a few sections of a single ovary. Therefore, a very careful analysis and critical consideration of the evidence must be made; even with this, the end result is an interpretation and not an absolute demonstration. For example, probably not a single ovary could be found, from a few days after birth to adult life, in which primary follicles were not present, and the universal presence of a few small primary follicles might be interpreted as their persistence in a latent condition from the embryo to adult life. Also it is impossible to identify each oocyte and primary follicle and positively demonstrate just when and where it had its origin; in structure and appearance each primary follicle resembles every other one, and other means must be sought to determine its pedigree. Still further, there is nothing in a ripe graafian follicle itself which indicates whether it has grown rapidly from a recently arisen oocyte, or represents a slow growth of a long latent oocyte formed in the early embryo. All this complicates the problem and renders more difficult a logical and satisfactory conclusion, though, fortunately, it does not make the solution impossible.

In evaluating the evidence precautions are essential: observations on a few ovaries or on ovaries of widely separated stages are not sufficient, there must be a close series of ovaries from early embryos to adult life; scattered observations on each ovary as a distinct entity are largely useless; consideration of any single phase of origin, growth, or degeneration, even of the whole series, is not ample. Observations upon all phases of the entire series must be considered as a unit taken together; only thus may one properly allocate the individual items and reach a balanced opinion.

Taking the observations as a whole, they are so consistent and so in harmony with the hypothesis of Edgar Allen and Papanicolaou that they furnish strong support for the view. Nor do contrary views seem to explain the facts so well. It is almost a matter of general agreement that early growth of ova in prepubertal life occurs in mammals and is followed by widespread degeneration of ova and follicles before the time of sexual maturity. There is also accumulating so much evidence of the continued origin of ova in late prenatal and adult life in various mammals that this can hardly be doubted to be a fact. It seems to me, further, that the view of long persistence of latent or resting follicles is based on tradition and assumption rather than on carefully ascertained evidence derived from investigations of the question itself. Taken by itself it must be admitted that one cannot prove that no follicle remains latent and after months or years again renews its development; just as one must admit that some primary follicles can always be found in any prepubertal and adult mammalian ovary. But such observations simply cannot be considered by themselves, they must be taken with the sum of other lines of evidence. If there be such excess of ripening of ova in graafian follicles beyond the needs of the animal at each oestrus, which seems to be generally true; if there be continued production of ova and new follicles in many mammals, which can hardly be doubted, how else can it be logically explained except by continuous growth and development? Assuming that many latent primary follicles are stored in the mammalian ovary, a serious difficulty is encountered in explaining the stimulation of a few to renewed development, while others remain quiescent. Likely enough, some hormone might stimulate renewed activity, but why of a few and not all of the same age? On the contrary, if the young follicles in an ovary at any one time represent different generations, if they have been formed at different periods, they are simply in varying stages of a continuous growth process.

A comparison of the germ-cell cycle of male and female mammals is helpful. In males the indifferent cells of the seminiferous tubules (spermatogonia) continue to grow and divide throughout life, and spermatocytes once formed do not seem able to remain latent, but must complete their development or die. On the other hand, in the female it is assumed that similar cells (the oocytes) have been formed at the same time once for all and may remain quiescent for extremely long periods. If oogenesis can be shown to be a continuous process, as is true of spermatogenesis, the female germ cells would be in agreement in this just as they are in maturation and other phases. Such marked differences in the gametogenetic cycle of the sexes is not characteristic of most animals, it has been ascribed only to the higher vertebrates; some of the observations presented by the authors referred to in this paper suggest doubt as to whether this difference is actually true of mammals.

There is still another similarity between oogenesis and spermatogenesis in the prepubertal gonads. It has been shown above that there is a ripening of ova at several periods before sexual maturity, i.e., before such ova can be functional; in another place (Hargitt, '26) I have shown that spermatogenesis also begins long before sexual maturity. Primary spermatocytes appear as early as twenty-three days after birth and some of these degenerate; spermatids are found at thirty-three days; between this and forty-three days several generations of spermatids are produced and undergo degeneration. Only by forty-eight days after birth are spermatozoa formed in small numbers and abundant, perfect spermatozoa, about fifty-five days. Therefore, in the male as well as in the female, the gametogenetic cycle begins long before sexual maturity and several generations of developing gametes degenerate and disappear before functional ones are produced. If several lines of evidence indicate close similarity in the germ-cell cycles of male and female, this may be considered as at least adding weight to the view of a similarity in the processes of origin and growth as a continuous process in the two sexes.

The material on which this study is based covers abundant embryonic and prepubertal stages with but few adult ovaries. I have emphasized this, because it has been omitted or only incidentally touched by others; the careful study of adult ovaries by Edgar Allen ('23), Allen, Kountz, and Francis ('25), Papanicolaou ('24), and Butcher ('27) add the needed observations for the adult stages. However, I am not able to decide whether the new formation of ova and their degeneration is a process repeated at each oestrous cycle (Edgar Allen), or is a continuous process as suggested by Paladino, Arai, Papanicolaou, and Butcher; my observations tend to confirm the latter view.

It seems to me that the question raised and the hypothesis presented by several investigators, and discussed in this paper, is an important one. Since it raises an issue not previously emphasized, further study is needed, and only such consideration can determine which view contains the greater amount of truth. With the progress of the present study I have been impressed by the fact that the observations seem more logically and clearly to support the view of the continuous formation of ova throughout the reproductive period of the female mammal. This involves not only new formation of ova, but continuous degeneration of ova, and the improbability of any oocyte or primary follicle remaining latent for a long time. Each oocyte seems impelled to proceed at once with its development just as is true for each spermatocyte.

It is hoped that the present report may serve as a contribution to the solution of the problem.

SUMMARY

The general opinion seems to be that ova of higher vertebrates are formed in the embryo once for all, and after birth no new ones can be added; most of these persist for long periods (even for years) in a latent condition, and at periodic intervals throughout adult life a few resume their activity and constitute the functional ova. Several authors have presented an alternative hypothesis: The whole oogenetic process is a continuous one from the embryo to the cessation of sexual activity in the adult. New ova are constantly being formed from the germinal epithelium of the ovary and once formed must begin and proceed with their development; no oocyte or primary follicle can remain in a latent condition, but must grow or die.

An examination of the life cycle of several female mammals discloses the following facts: 1) Shortly after birth practically all ova begin their growth at the same time, reach maturity and form polar bodies before puberty, few oocytes or follicles remaining in the ovary. 2) During the same period a profound degeneration occurs and increases until most follicles have disappeared before puberty. 3) The number of oocytes and primary follicles decreases and new formation of ova nearly ceases by twenty-three days after birth in the rat; then, until the time of puberty new ova are formed in large numbers and these at once begin their growth, development, and maturation. 4) New ova are formed from the germinal epithelium of adult ovaries and are constantly moving into the ovary to replace those which have degenerated or been discharged. 5) These new ova originating in the adult ovary pass through the same continuous process of growth, maturation, degeneration or ovulation.

When these several facts are analyzed, they are found to be entirely consistent with each other and constitute a single line of evidence which strongly supports the view of continuous formation of new ova throughout life. Furthermore, this explanation seems to be more in accord with the ovarian history than the older view. Consequently, the alternative hypothesis merits careful consideration and investigation. Further work on other mammals is needed to fully confirm this view if it is to be established, though present evidence is not inconsiderable.

In the albino rat it seems probable that new ova are formed from the germinal epithelium of the ovary throughout life, from the embryo until late in adult life; that each ovum when once formed must at once begin its growth and development; that the majority of these degenerate and die and never come to ovulation; and finally, that oocytes and primary follicles do not remain latent for long periods, but pass through a continuous process resembling that of spermatocytes in the testis.

BIBLIOGRAPHY

Allen, Edgar 1923 Ovogenesis during sexual maturity. Am. Jour. Anat., vol. 31, pp. 439–482.

Allen, Edgar, Kountz, Wm. B., and Francis, B. F. 1925 Selective elimination of ova in the adult ovary. Am. Jour. Anat., vol. 34, pp. 445–468.

Arai, Hayato 1920 On the postnatal development of the ovary (albino rat), with especial reference to the number of ova. Am. Jour. Anat., vol. 27, pp. 405–462.

Butcher, Earl O. 1927 The origin of the definitive ova in the white rat (Mus norvegicus albinus). Anat. Rec., vol. 37, pp. 13–30.

Firket, Jean 1920 On the origin of germ cells in higher vertebrates. Anat. Rec., vol. 18, pp. 309–316.

Hargitt, Geo. T. 1925 The formation of the sex glands and germ cells in the albino rat. I. The origin of the germ cells in the albino rat. Jour. Morph. and Physiol., vol. 40, pp. 517–557.

——— 1926 Ibid. II. The history of the male germ cells in the albino rat. Jour. Morph. and Physiol., vol. 42, pp. 253–306.

——— 1930 Ibid. III. The history of the female germ cells in the albino rat to the time of sexual maturity. Jour. Morph. and Physiol., vol. 49, pp. 277–332.

Lange, J. Die Bildung der Eier und der Graaf'schen Follikel bei der Maus. Verh. phys.-med. Gesell., Würzburg, N. F., Bd. 30, S. 56–76.

Paladino, G. 1887 Ulteriori ricerche sulla distinzione e rinnovamento continuo del parenchima ovarico dei mammiferi. Anat. Anz., Bd. 2, S. 835–842.

——— 1903 Sulla rigenerazione del parenchyma ovarico e sul tipo di struttura dell' ovaja di Delfina. Rend. della R. Acad. delle Scienze Fisiche e Matematiche di Napoli, Fasc. 12, 42 an., pp. 289–298.

Papanicolaou, George N. 1924 Ovogenesis during sexual maturity as elucidated by experimental methods. Proc. Soc. Exp. Biol. and Med., vol. 21, pp. 393–396.

Pearl, Raymond, and Schoppe, Wm. F. 1921 Studies on the physiology of reproduction in the domestic fowl. XVIII. Further observations on the anatomical basis of fecundity. Jour. Exp. Zoöl., vol. 34, pp. 101–118.

Pflüger, E. 1863 Die Eierstöcke der Säugethiere und des Menschen. Leipzig.

Winiwarter, H. von, and Sainmont, G. 1908 Nouvelles recherches sur l'ovogénèse et l'organogénèse de l'ovaire des mammifères (chat). Arch. de Biol., T. 24, pp. 1–143, 165–276, 373–433, 627–652.

PLATES

EXPLANATION OF PLATES

All figures represent typical sections of entire ovaries; the outlines of the ovaries, follicles, and other parts are drawn with a camera lucida; the shading is diagrammatic. As reduced in the engraving, figures 1 to 3 are magnified about 80 times while figures 4 to 8 are × 43.

PLATE 1

EXPLANATION OF FIGURES

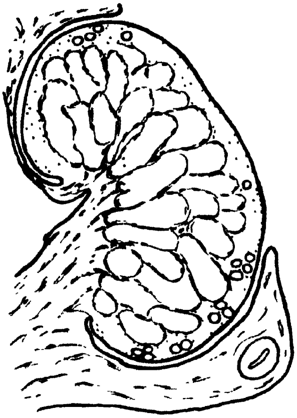
1 Embryo, twenty and one-half days. The ova are in radially arranged masses, their nuclei in synizesis; only the position of the cords is shown. There are germ cells in the germinal epithelium and tunica albuginea. Ovarian capsule forming.

2 Newborn animal. The cords or masses of ova are breaking up into 'egg nests,' their location represented by the unshaded part. Nuclei of ova all in synizesis or pachytene stages.

3 Three days after birth. Most of the egg nests have broken up to form primary follicles, shown by the two concentric circles; in the cortex nests still present.

4 Fourteen days after birth. All ova of earlier periods are about in the same stage of young growing follicles, a few of which are becoming vesicular. Oocytes occur in germinal epithelium and tunica albuginea.

5 Twenty-three days after birth. The majority of ova are in mature graafian follicles with the ovum in maturation. Many follicles are cut to one side of the center and show only in part. Some follicles are degenerating and contain fragmenting ova. Very few growing follicles and still fewer oocytes.



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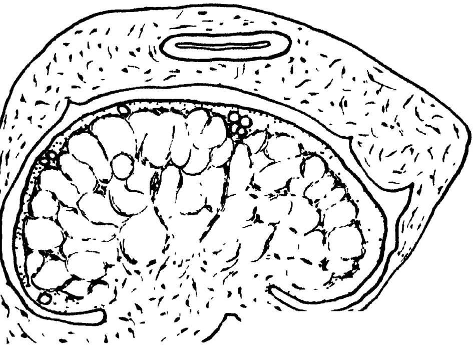

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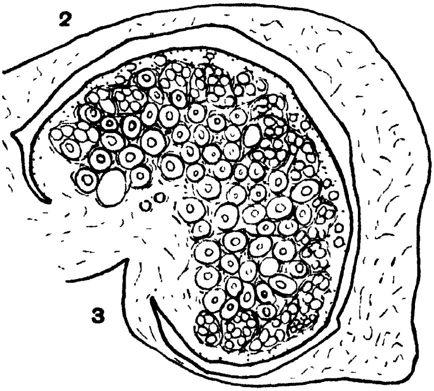

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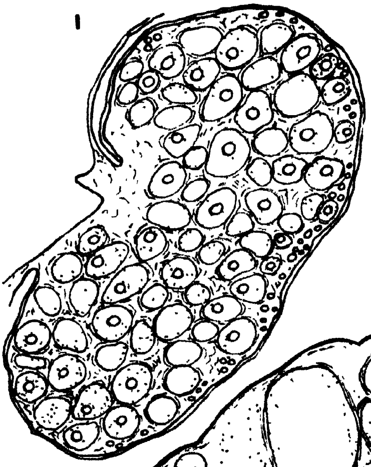

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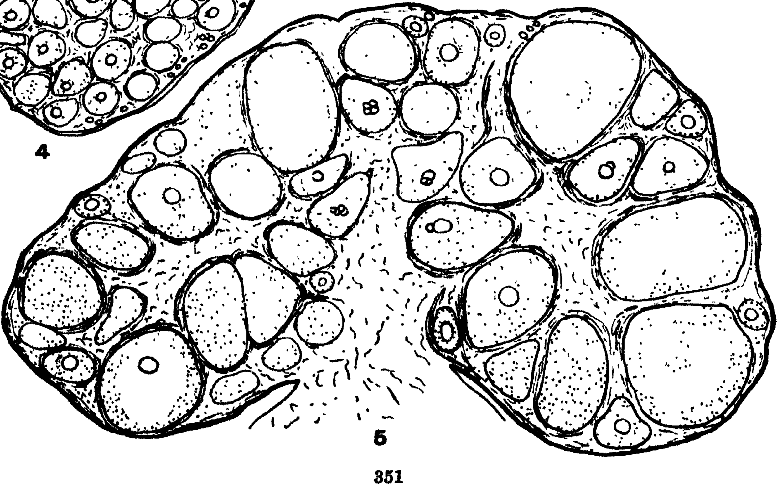

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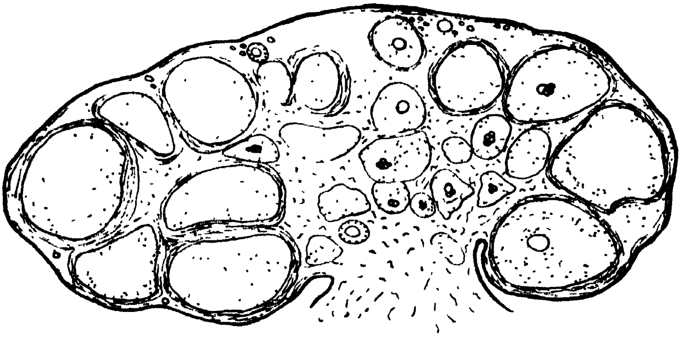
PLATE 2

EXPLANATION OF FIGURES

6 Twenty-nine days after birth. At this stage note again that most ova are in the same stage of development, mature graafian follicles with ova forming polar bodies. In center of ovary are remnants of many degenerate follicles. Oocytes and young follicles rare.

7 Thirty-nine days after birth. Only a portion of the section is shown. Most of the ovary is filled with masses of degenerated follicles, shown by the more densely shaded portions; a very few mature graafian follicles are still present. Oocytes and young follicles more abundant.

8 Forty-five days after birth. Only a portion of the section included. Ovulation has occurred and parts of three corpora lutea are present in this part of the ovary. The center of the ovary is filled with degenerate follicles. The ovary represents nearly the normal adult ovary with a few large graafian follicles and a few growing follicles; oocytes present in or below the tunica albuginea.



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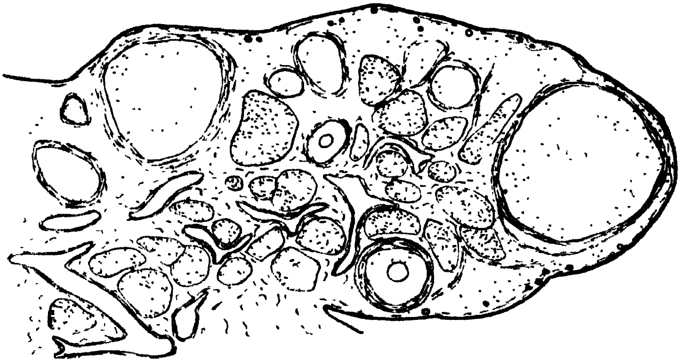

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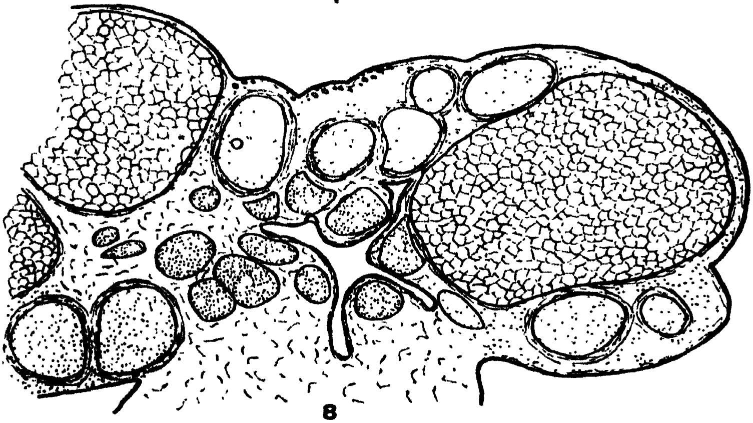

8

THE NATURAL HISTORY OF BUGULA FLABELLATA AT WOODS HOLE, MASSACHUSETTS, INCLUDING THE BEHAVIOR AND ATTACHMENT OF THE LARVA

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FIVE TEXT FIGURES AND TWO PLATES (THIRTEEN FIGURES)

AUTHOR'S ABSTRACT

The breeding season of Bugula flabellata extends from June 10th to November 15th. The young embryos develop in brood pouches (ovicells) and are finally expelled from the colony as swimming embryos. They come from the colonies at dawn or early morning. After a free-swimming period of four to six hours, each larva becomes attached and after a profound metamorphosis which involves the loss of larval organs, it develops into the bryozoan colony by budding.

The larvae at first are positive to light, but become negative before attachment. Their behavior is described in detail and the mode of attachment is explained.

After the larva has become attached a period of rapid growth by budding ensues. The rate of growth is given in a table in which it is shown that the first individual of the colony is completed in two days and that a new series of buds is formed every two days. There are eight or ten individuals after one week and over a hundred in two weeks. In one month the colony is half-grown and becomes sexually mature. A colony becomes senescent in three months, when it measures 1½ to 1¾ inches in diameter.

Younger colonies hibernate successfully and resume growth in early May, when new polypides are formed.

POLYZOA IN GENERAL

The Polyzoa or Bryozoa received recognition as a separate phylum of animals about 1830, due to the anatomical studies of J. V. Thompson. The life history of those species of Bryozoa whose larvae develop in brood pouches, known as ovicells, was fairly well understood by 1860. Huxley had shown, in 1856, that the eggs and spermatozoa of certain species are produced within the coelom, from which, after self-fertilization, the egg passes into the ovicell and there develops into a ciliated larva. It was not known that a few species of Bryozoa extrude unfertilized eggs until much later (Prouho, '92). Schneider ('68), in a remarkable one-page paper, explained in its main outlines the nature of the metamorphosis of the larva into the attached colony.

Therefore, as a composite result of this early work, it was shown that the life history of the Bryozoa includes a free-swimming larva which finally becomes attached and, after a profound metamorphosis, develops by budding into the bryozoan colony. The larva has a complicated structure of transitory organs which disintegrate during metamorphosis.

Of the early papers dealing with the life history of Bryozoa, those by Van Beneden, Huxley, Schneider, Nitsche, and Barrois are perhaps the most important. More recent papers of equal merit are those by Prouho, Seeliger, Kupelwieser, Calvét, and Harmer.

Remarkable advances were made in embryology and in the interpretation of developmental stages after methods of sectioning and staining had been introduced about 1870. One result of applying modern technique by these workers was to show that the larva of the Bryozoa closely resembles the trochophore of annelids and Mollusca. The bryozoan larva, however, usually has only a rudimentary digestive tract or none at all. This is undoubtedly due to the parasitic nature of the larva from its habit of developing in a marsupium. The chief constructive result of the more recent studies has been to explain, first, the details of early development; secondly, the structure and function of the larval organs, and thirdly, the exact nature of metamorphosis and its similarity to ordinary asexual budding in the growth of the colony.

The contribution of the present writer is mainly concerned with a study of the breeding season, the behavior and attachment of the larva, the rate of growth of the colony, and its age at sexual maturity. However, a careful study of the entire life history covering a period of five years has been made. Except for casual observations on the attachment of the larva, almost nothing has been written on these subjects.

ECOLOGY

Two species of Bugula, turrita and flabellata, are abundant in the waters about Woods Hole. They differ in the shape of the colonies, but are otherwise similar in their structure and life history. This paper is based upon a study of B. flabellata, but significant differences between the two species are described in so far as they have been observed. When not otherwise stated, B. flagellata is indicated.

Bugula turrita occurs exclusively in Vineyard Sound. It was not found in Eel Pond. Bugula flabellata, on the other hand, is abundant in Eel Pond and is not found outside.

Vineyard Sound and Eel Pond communicate freely and their tides are approximately equal. However, the tidal currents in the Sound are very much stronger than those in the relatively sheltered Eel Pond. Although the two situations studied are only 100 yards apart, it is evident that the biological conditions differ considerably, as shown by the further fact that, when a colony of either of these species is transferred to the habitat of the other, it does not thrive, and after a few months or a year disappears.[1] It has also been shown that Teredo navalis is much more abundant at the Cayadetta dock in Vineyard Sound than in Eel Pond. There is likewise a difference in the species of hydroids which inhabit the two localities.

Bugula flabellata occurs principally upon the lower surfaces and edges of horizontally submerged wooden timbers or on the shaded portions of vertically placed structures—a phenomenon to be explained later.

Bugula flabellata is not hardy in this climate. It appears to be the rule that mature colonies do not survive the winter, especially when they are in exposed situations where waves may tear them to pieces. The younger colonies, less than half grown, fare better, being more vigorous and having less surface exposed to wave action. The smallest colonies, including those that are established after September 25th, are the most resistant.

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[1] Bugula turrita, attached to a floating dock, transferred from Vineyard Sound to Eel Pond in September, 1926, successfully survived the winter, but other attempts to transfer it failed. The several attempts to transfer B. flabellata all failed.

There are, so far as observed, no active polypides after the first of November. They hibernate as cysts or brown bodies which have every appearance of being true cysts, but it has been shown that they do not regenerate into polypides. The colonies remain inactive until the first of May—long after most animals have been actively feeding and developing.

THE BREEDING SEASON

Neither species of Bugula extrudes eggs: the embryos develop in special brood pouches known as ovicells, each of which contains a single embryo. The young leave the parent colony as ciliated free-swimming larvae. B. flabellata begins to breed either the first or second week in June and continues until late in November. In 1925, the first larvae were liberated about June 5th, but in 1926, not until June 15th. There is reason to believe that the second week in June is the usual time for the opening of the breeding season of this species at Woods Hole.

Numerous experiments show that larvae metamorphose successfully between November 1st and November 20th. These mostly remain too small to be seen by the unaided eye and pass the winter as colonies of from one to eight individuals. They resume growth during the first week in May and come to sexual maturity by the middle of June. The latest date on which larvae were found was December 1st (1925) and November 26th (1926). These late-November larvae probably do not succeed in metamorphosing and establishing themselves as young colonies. No colonies have survived the winter upon test blocks placed in the water after November 20th.[2]

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[2] The larvae that were observed late in November and early December were sluggish in their movements, but as the water warmed, after being placed in the laboratory, a few were seen to leave the colonies and swim not too vigorously. During the years 1925 and 1926 visits were made to the laboratory during every month in the year, February excepted, to make observations on the condition of these species and other animals under observation. Otherwise complete studies of natural history could not be made.

The extreme limits of the breeding season of Bugula flabellata may therefore be given as June 1st to November 20th, and usually June 10th to November 15th. This species of Bugula therefore has a comparatively long breeding season and the larvae are active through a wide range of temperatures.

There is apparently a high mortality among the larvae liberated at the close of the season. When the water cools with the approach of winter, they become too sluggish to find a support and therefore fail to complete their development. The very last larvae to metamorphose are usually attached to older colonies and pass the winter as single individuals. They are established from November larvae, and they and other small colonies, up to eight or sixteen individuals, are the first to resume growth in the spring. It is possible that some of the latest larvae metamorphose within the ovicell and there hibernate, since new colonies sometimes are observed to spring from the branches of old ones.

THE LARVA

The larvae of both species of Bugula are almost spherical in form. The average size is 0.17 to 0.19 mm., but individuals having a diameter of 0.30 mm. have been observed. A prominent apical organ occurs at the anterior end of the larva.[3] Its external surface is convex and is separated from the body of the larva by a circular depression. It is otherwise clearly defined by lack of color and glandular appearance, the body being yellowish. A circlet of large rigid cilia or bristles surrounds or borders the apical organ (fig. 1). A well-defined groove extends across the posterior end of the larva and forward on one side to the equator. This structure should perhaps be described as consisting of two parts, since the posterior depression is incompletely separated from the lateral groove. The depression at the posterior end is circular and contains in its center an invagination known as the internal sac (fig. 7). The lateral part of the groove is narrow and is bordered by two prominent ridges or lips, whose walls contain a gland (figs. 1, 3, and 7). Within the anterior end of the groove, in the region of the equator, a tuft of large active flagella is found (fig. 1). It consists of ten or twelve flagella which beat back and forth as a unit in an anteroposterior direction. No other structures are visible in the living larva of Bugula flabellata, but the larva of Bugula turrita has, in addition, darkly pigmented eyespots symmetrically placed with reference to the lateral groove (figs. 5, 6, and 7). Two eyespots are located on the side opposite the groove in the anterior hemisphere, and are perhaps 100° apart (fig. 5). Similar eyespots, in some cases two, in others four, occur at the anterior margin of the lateral groove. These are also symmetrically placed with reference to the groove, but very close to it (fig. 6). A study of the behavior of the larva indicates that the eyespots and the tuft of flagella function in orientation to light. They will be described in another section of this paper. The internal sac has long been known to be the attaching organ, but no function has been certainly assigned to the lateral groove and its associated structures, collectively known as the piriform organ. The figures of the larvae, plate 1, all of which were drawn with camera lucida, show the external features just described. The larva of Bugula turrita which has the pigmented eyespots is slightly larger and more variable in size than that of Bugula flabellata and it is somewhat more sensitive to light.

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[3] This refers to the end that is carried foremost in swimming and not to the morphological anterior end.

A general similarity in both structure and behavior characterizes the larvae of Bryozoa that have been described. A glance at the figures by Barrois ('77) and of Calvét ('00) is convincing on this point, although the variety of external form is remarkable. The larvae of species possessing brood pouches have rudimentary larval digestive organs or none at all, and the larval period is much abbreviated, while those species which extrude eggs into sea-water have a larva which resembles a trochophore, including a complete digestive tract and an extended larval period. Bugula belongs to the former group.

French students, who have written most extensively upon Bryozoa, refer to the posterior depression as the 'oral depression' because it contains the mouth, when one is present, as well as the internal sac. They call the lateral groove with its associated structures the 'piriform organ.' The tuft of large flagella is known appropriately as the 'vibratile plume.' The term piriform organ has been used indefinitely. Sometimes it is meant to refer to this whole structure, sometimes to the gland in its walls, and finally, Barrois, who introduced the term, confused it with still other structures, as was shown by Prouho in his detailed study of the structure and development of the larva. The latter considered it mainly a sense organ.

The larva of the European Bugula flabellata, as drawn by Nitsche, is distinctly different from our American form under consideration, in that the European form has numerous pigmented eyespots. It would be interesting to know if these are in reality the same species. Calvét also speaks of a confusion of varieties. It should be noted further that two European species, Bugula flabellata and avicularia, are identical in the form and appearance of their larvae, while the adult colonies differ perceptibly. This is shown both by the figures and description as given by Nitsche and Barrois. They also figure the larva of Bugula plumosa with four eyespots placed precisely as in my figures of the larva of B. turrita as shown in my figures 5 and 6. These unexpected similarities between the larvae of the various species may be of speculative interest to the morphologist as indicating possible kinships and interrelationships not shown by the adult colonies.

BEHAVIOR OF THE LARVA

Liberation of larvae

The larvae of Bugula flabellata emerge from the colonies in the early morning, beginning at dawn, and continue to appear in increasing numbers until 9 or 10 o'clock. After that hour the numbers liberated diminish rapidly. No larvae are liberated at night and very few in the afternoon. In order to obtain them in quantity, mature colonies of this species are collected late in the afternoon and placed in bowls of sea-water before a window, where they remain overnight. Larvae will be found swimming in the bowl by sunrise and may be taken up with a pipette. For a study of their behavior they were placed in Stender dishes or rectangular dishes approximately 2½ inches in diameter.

From colonies kept in a dark room overnight larvae are not liberated at dawn and not until they are removed to the light. This experiment, repeated several times with uniform results, shows that light is the stimulus which brings about the liberation of the larvae.

Mode of locomotion and orientation

The body of the larva is densely covered with small locomotor cilia, except at the anterior end.[4] These cilia beat obliquely backward and cause the larva to rotate on its axis to the left as it swims forward (fig. 3). The tuft of giant cilia (vibratile plume) in the anterior margin of the lateral groove beats back and forth in an anteroposterior direction and causes the larva in its swimming ordinarily to swerve from a direct course. The path followed, therefore, is usually a spiral, sometimes wide, sometimes narrow. When swimming in a spiral path, the lateral groove is on the outside of the spiral. This leads to the conclusion that the forward movement and rotation of the larva are due to the beating of the small cilia, and that the swerving from the direct line is due to the action of the tuft of large flagella (vibratile plume). The mode of stimulation of the eyespots and vibratile plume was not studied, but these structures are no doubt concerned in the orientation of the larva to light and shadow. Practically all parts of the piriform organ contain nerve cells which indicate that these structures function in part as a sense organ. This region has also been shown to be directly connected by nerve fibers with the apical organ, some elements of which appear to constitute a ganglion (Prouho, '92; Seeliger, '06; Calvét, '00).

Reactions to light

The larva is an active swimmer and when first liberated is strongly positive to light. It later becomes negative in its heliotropic response. Experiments were made repeatedly which showed this reversal of response to light to be the rule. The cause of this reversal of behavior is obscure, but its utility is evident in that it results in the ultimate dispersal of the larvae.

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[4] I call the end which is carried foremost in swimming the anterior end, without regard for the morphological anterior end.

Table 1 shows that the positive response of the larva to light is at first very pronounced, but that it lasts only two or three hours. In many similar experiments the positive response to light was 100 per cent, and the change to the negative response a few hours later was equally complete.

There is no difficulty in recognizing the positive character of the response of the larva to light when it first emerges from the parent colony, but the change to the negative response to light which ensues two or three hours later might be overlooked. The change from a positive to a negative response comes on gradually. If a single larva is followed in its movements at this period, it is seen to swim from the lighted side of the dish toward the dark side, but before reaching the opposite side, the response to light again becomes positive and it returns to the illuminated side of the dish. The larva is not, therefore, at this time indifferent to light, but is alternately positive and negative. During this intermediate period the larvae are swimming back and forth, with about equal numbers collected at the most illuminated and least illuminated sides of the dish. Shortly after this, they become predominantly negative in their response to light and collect in greater numbers on the least illuminated side of the

TABLE 1

Behavior of the larva of Bugula flabellata. Larvae extruded 6 to 8 A.M.*, September 10, 1926. Table showing the rate of change from positive to negative response to light*

HOUR	NUMBER OF LARVAE IN MOST ILLUMINATED HALF OF THE DISH	NUMBER OF LARVAE IN LEAST ILLUMINATED HALF OF THE DISH
8 A.M.	22	6
9 A.M.	18	10
9.30 A M.	10	18
10.30 A.M.	2	26
2 P.M.	2	26

dish. They now begin to attach and undergo metamorphosis. It is noteworthy that alternately positive and negative responses persist during the latter part of the free-swimming period. The larvae continue intermittently to make excursions toward the source of light until they finally become permanently attached, although the radius of these excursions becomes shorter and shorter.[5] One cannot avoid the observation that this reaction has utility in that it adds to the chances that the larva will, in its systematic wanderings, ultimately find a suitable support for the developing colony. As further evidence that this is a correct inference, attention is called to the fact that the negative reaction to light enables the larva ultimately to find a solid support. While studying larvae with a microscope, it was noted that they may be driven to any desired part of the field by changing the position of the source of light or by using the hand to throw a shadow in various places on the field. While the larvae are strongly positive to light they quickly seek the lighted part of the field, and when strongly negative to light they as quickly turn toward a shadow. Some authors have called this a shadow reaction. Its utility is shown by the fact that when, in swimming about, they come near an object that casts a shadow, they become at once oriented toward it and thus find a support. This shadow reaction accounts for the abundance of Bugula flabellata on the lower surfaces of timbers and their absence on the upper sides—a fact previously mentioned in reference to the ecology of this species. It was noted that the barnacle Balanus eberneus is found in the same situations and we may suppose that the larva of this species behaves in the same way.

In order to test the reactions of the larvae to light with precision, a series of experiments were made in a dark room where the direction and intensity of light rays could be controlled. Light was admitted through a small window which could be opened to any desired extent. No artificial light was used in these experiments.

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[5] Calvét ('00) observed that a larva swimming in a glass of sea-water selects a place upon the glass where it chooses to metamorphose and returns to this spot repeatedly after swimming in circles. It finally becomes attached at this spot. Nitsche ('70) also noted that the larvae do not swim constantly, but make frequent momentary attachments.

Without describing the apparatus in detail, the results of the experiments may be given in a few words:

1. When light was allowed to fall directly and horizontally upon a dish of sea-water containing numerous swimming larvae of B. flabellata, 80 to 95 per cent of them in time became attached to the side of the dish away from the source of light.[6]

2. When an opaque shade was placed between the window and the dish of larvae and a mirror was used to reflect the light at an angle of 90° upon the dish of larvae, they attached in accordance with the source of reflected light, i.e., on the side of the dish farthest from the mirror.

Numerous variations of these experiments were made and all showed conclusively that larvae become negative in their response to light before attachment.

Response to gravity

The reaction of the larva to gravity is negative, since, when placed in total darkness, it invariably attaches at or near the surface of the water. This continued to be true even when a free air surface was avoided by inverting the dish under water. In nature, however, its negative response to sunlight is able to drive it below the surface, because colonies of B. flabellata are found not at the surface only, but attached to piles at all levels between the bottom and the upper surface. Laboratory experiments designed to test this assumption were not uniformly successful. In only one of five experiments was I able to drive the larvae to the bottom for attachment, by directing light rays upon them from above. It is obvious, however, that in nature the negative response of the larva to light at the time of attachment is the effective one.

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[6] Nitsche ('70) made the observation that larvae kept in a laboratory become attached to the side of the dish nearest the window—which observation must be an error.

The following diagram shows the arrangement of the apparatus used in this series of experiments. In one case in which the light was directed obliquely downward upon the larvae, they were driven to the bottom and they metamorphosed either at the bottom or at some distance below the surface. The failure of the other experiments is unaccountable. The fact that one experiment gave the expected result indicates that the interpretation given is the correct one; i.e., that the negative response to light at the time of fixation is stronger than the negative response to gravity.

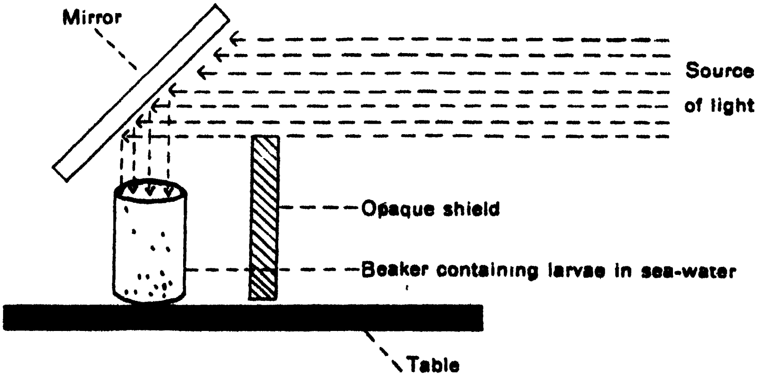

Diagram of apparatus used in dark-room experiments on reactions of Bugula larvae to light

Duration of the free-swimming period

Several experiments were carried out to determine the duration of the free-swimming period.[7]

It was shown repeatedly that the duration of the swimming period of a majority of the larvae is from four to six hours, with four to eight hours as extreme limits. The larvae attach more promptly when exposed to direct light of moderate intensity than when in very dim light. Attachment is very considerably delayed or prevented altogether if the larvae are kept in absolute darkness. This is explainable on the ground that they are accustomed, in nature, to the influence of light and shadow in finding a supporting surface. In fact, they are driven to the support by something resembling a tropism. Table 2 gives the results of an experiment in which one dish of larvae was placed on a laboratory table in front of a window, one in a dark room near a small window which admitted light, a third in this same dark room but ten feet from the window so that the amount of light reaching it was considerably reduced, and a fourth in total darkness. By reference to the table, it will be seen that the larvae in no. 1 and no. 2 became attached more promptly than those in nos. 3 and 4. It is also shown that some larvae failed to meta-

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[7] Calvét ('00) observed the metamorphosis of the larvae of several species of Bryozoa and concluded that the free-swimming period varies from fifteen minutes to six or eight hours.

TABLE 2

Behavior of the larvae of Bugula flabellata. Tabulation showing the duration of the free-swimming period of the larvae and rate of attachment under various conditions of lighting. Larvae liberated 7 to 8 A.M., September 18, 1926; temperature, 19.5°C.

EXPERIMENT. WHERE PERFORMED	11 A.M.	12 M.	1 P.M	2 P.M.	3 P.M.	4 P.M.	
	Attached	Attached	Attached	Attached	Attached	Attached	Free swimming
1. On laboratory table	10	32	48	56	56	57	16
2. In dark room near open window	0	52	69	78	80	80	8
3. In dark room, 10 feet from window, dim light	0	23	29	..	35	37	20
4. In dark room with no light	0	0	0	14	..	18	21

NOTE: This table shows that larvae that are shed between 7 to 8 A.M. begin to attach between 11 and 12 A.M., and have nearly all become fixed by 2 P.M. It also shows that larvae metamorphose more promptly in a moderately lighted room than in a totally dark room. A few larvae fail to attach under any conditions, but more fail to settle permanently in the dark than in a room that is lighted. (See right-hand column of table 2.) The duration of the free-swimming period is shown to be from four to eight hours, and for the most part from four to six hours. This was verified by numerous experiments.

morphose under any of the conditions. This was observed many times. In every group of larvae there are a few that never become fixed. They continue to swim for a day or two and finally die.

Mode of attachment and metamorphosis

After a swimming period of from four to six hours, as stated above, the larva becomes attached, and within another two or three hours it has elongated to 0.25 mm. and has broadened out at the base to form an attaching disc or holdfast. The mode of attachment of both species of Bugula was observed repeatedly with the compound and binocular microscopes. No difficulty was experienced in inducing the larvae to complete their metamorphosis in a small dish of water and many were seen to make the permanent attachment and quickly form the disc-like holdfast. Every detail of the process was observed and a clear idea of the function of the so-called piriform organ (lateral groove and vibratile plume) was gained, so far as these species are concerned.

Mention has already been made of the posterior depression and lateral groove of the larva, and it was stated that the posterior depression contains an inverted or internal sac, which is the attaching organ (fig. 7). The lateral groove with its tuft of large flagella is undoubtedly concerned with the orientation of the larva during its swimming existence, but it also takes a part in metamorphosis, so that a discussion of its function has been reserved for this section. That it is in part a sense organ which has to do with surveying a suitable attaching surface, as suggested by Prouho ('92), is evident. The swimming larva, even when first liberated and while still strongly positive to light, has a habit of adhering momentarily to any surface with which it comes into contact. Its swimming is therefore not continuous, but is interrupted by frequent temporary attachments. The larvae of Bugula turrita when most sensitive react instantly to light, and by cutting off the direct light from the window to the stage of the microscope, they were attracted at once to the field illuminated from below by the mirror. They were thus readily observed under the microscope in considerable numbers as they swam into the illuminated field and attached to the slide. It was noted that the ridges on either side of the groove were contractile and that they were alternately being extended and then retracted to the extent of obliterating them (compare figs. 3 and 4). It was readily seen that the larvae grasped the slide by means of these ridges, which seemed to be more or less glandular and adhesive as well as muscular. It is apparent then that the lateral groove is used in part as a temporary attaching organ. Larvae in which the process of permanent attachment was observed first formed a temporary attachment as just described, then suddenly the internal sac was everted in contact with the slide to form the permanent holdfast. This remarkable organ is everted with great suddenness and when fully extended it appears to be almost as large as the larva itself. It is frilled at the edge and is hollowed out like the base of an egg cup. This structure grasps the slide so suddenly and the larva becomes transformed in shape from a sphere to that of an hourglass so quickly that the sequence of changes is difficult to follow. It all happens within a second or two, so that one has to watch the process several times to get the details correctly.

As soon as the holdfast has anchored the larva, the lateral groove organ releases its hold. After permanent fixation occurs, both the cilia and the vibratile plume continue to beat for about twenty minutes and then cease to be active.[8]

The whole process of fixation is over within a second or two and further changes are the slow processes of growth. For a short time after attachment the metamorphosing larva remains spherical, but elongation is discernible within an hour. After four to eight hours the body cavity is clearly visible, and the rudiment of the developing polypide can be recognized near the tip of the elongating mass (figs. 8 and 9). It is said to develop from the invaginated apical organ, but this cannot be made out with certainty without a study of stained sections. (See Prouho, '92, for a detailed study of metamorphosis.) In a few hours more, however, zooecium or body wall and the polypide are clearly recognizable as such. In twenty-four hours the first individual of the colony appears to be fully formed, although it does not extend its tentacles and feed until about thirty-six or forty hours have elapsed (fig. 10). In his study of the European form of Bugula flabellata Nitsche gives five days as the time required for the development of the first person of the colony. This difference in rate may, however, be due merely to a difference in temperature.

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[8] Barrois ('79) gives a diagrammatic figure of the internal sac everted.

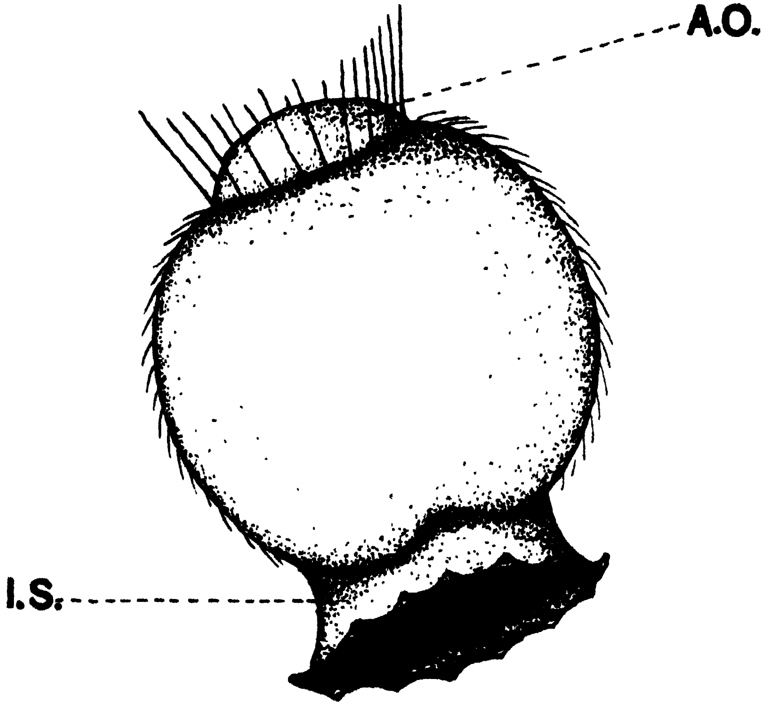

Fig. A A diagrammatic drawing to show the internal sac everted in the act of attaching. *i.s.*, internal sac; *a.o.*, apical organ.

Observations on the eversion of the internal sac or holdfast were greatly facilitated by the fact that when studying them with a microscope with substage illumination, the larvae, in accordance with their habit at the moment of fixation, turn so that the apical organ faces toward the light. The internal sac was therefore extruded directly upward and attachment was made to the surface film. A deep shadow thrown upon a larva at this time will cause it to turn about as described and extrude the holdfast. Contact with the substratum constitutes a part of the stimulus to extend the holdfast, which thus happens only when it will find a support to cling to. The first act of metamorphosis is to grasp the surface of the support by means of the lateral groove, or piriform organ, and this is followed by a spasm which extends the relatively enormous organ of fixation (internal sac). From this time the rapid development of the colony by budding ensues.

THE RATE OF GROWTH OF BUGULA FLABELLATA

As stated above, the larva of Bugula is nearly spherical and measures from 0.17 to 0.19 mm. in diameter. After a short swimming period, most of the larvae become attached. Then follows a period of rapid growth, and asexual reproduction by budding which results in the production of the colony.[9] Four or five hours after attachment, the metamorphosing larva has elongated to approximately 0.25 mm. and broadened at the base to form an attaching disc (fig. 8). As shown by table 3, the young zooid is now somewhat more slender than the larva, so that increase in length is not a sure indication of growth. In this case it is more in the nature of a change in form, although growth is beginning at this time.

From eight to twelve hours after attachment, the developing zooid has elongated still more and growth has clearly

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[9] The following terminology is needed in following the description as given in the following pages. 1. The zooid is one individual of a colony, including zooecium and polypide. 2. The zooecium is the body wall of the individual and it encloses the movable polypide as in a sheath. 3. The polypide includes the tentacles and digestive tract and is the retractile part of the zooid. 4. The coelom lies between the zooecium and the polypide.

TABLE 3

Rate of growth of Bugula flabellata

DATES	AGE	MEASUREMENTS OF COLONIES		NUMBER OF INDIVIDUALS IN COLONY	AGE AT SEXUAL MATURITY
		Length	Breadth		
		Mm.	*Mm.*		
July 8	Newly hatched larva	.17 to .19	.17 to .19		
July 8	1 hour	.165 to .18	.165 to .17		
July 8	2 hours	.21	.15		
July 8	5 to 7 hours	.23 to .25	.13 to .14		
July 8	8 to 12 hours	.31 to .40	.13 to .14		
July 8 to 9	1 day	.74 to .9	.16 to .17		
July 8 to 10	2 days	.8 to 1.1	.17 to .18	1	
July 8 to 11	3 days	1.35 to 1.6	.17 to .18	1+	
July 8 to 12	4 days	1.6 to 1.7	.17 to .18	2	
July 8 to 14	6 days	2.17 to 2.2	.7 to .8	4+	
July 8 to 16	8 days	3 to 3.1	1 to 1.1	8 to 10	
August 8 to 18	10 days	3 to 3.1	1.5 to 1.7	16 to 20	
July 15 to 27	12 days	4.2 to 5	4 to 5		
July 1 to 15	14 days	4.6	6		
August 13 to 27	14 days	5	6	100 to 110	
August 13 to 27	14 days	5	6.5	120 to 130	
August 13 to 27	14 days	5	6.75	150 to 170	
June 25 to July 10	15 days	5	6	85 to 90	
June 30 to July 15	16 days	6	9.2		
July 15 to August 1	16 days	6 to 6.5	9.5 to 10		
July 25 to August 12	18 days	7 to 8	10 to 12		The first larvae are expelled after 28 to 30 days
August 13 to September 3	21 days	7 to 8	11 to 12		
July 15 to August 12	28 days	12 to 13	20 to 22		
August 15 to September 15	30 days	12 to 13	20 to 21		
June 5 to July 8	34 days	13 to 15	20 to 23		
June 18 to July 22	34 days	15 to 16	23 to 25		
June 5 to July 25	50 days	22 to 23	32 to 34		Larvae abundant in six weeks
June 23 to August 12	50 days	22 to 23	32 to 35		
June 23 to August 23	60 days	22 to 25	35 to 38		
June 23 to August 23	60 days	1 inch	1½ inches		

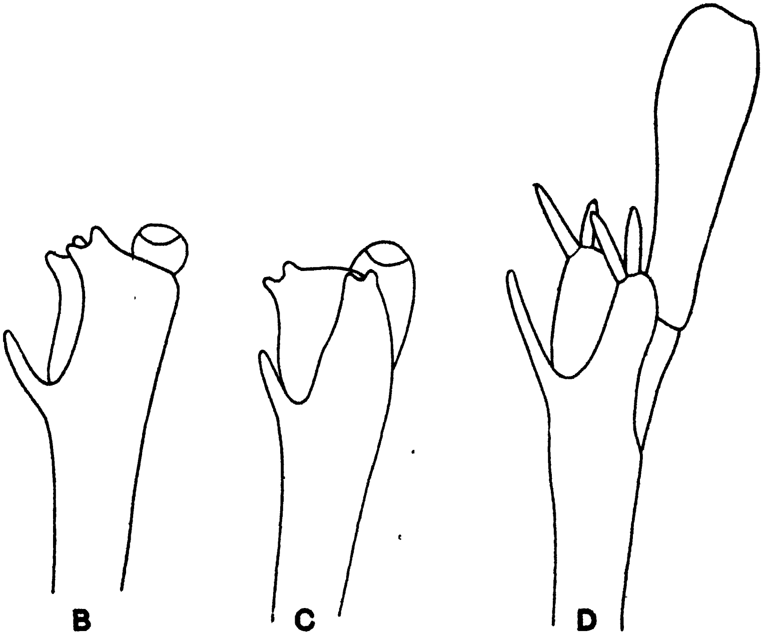
Note: The data of this table are not averages, but measurements of actual colonies. The differences in rate of growth are largely due to differences in the temperature of the water.

begun. A small spherical sac within the young zooecium is the anlage of the polypide, which already, at ten hours, has distinguishable tentacles. At this time it measures from 0.30 to 0.40 mm. in length and 0.13 to 0.14 mm. in thickness (fig. 9).

At the end of twenty-four hours, the first polypide is clearly recognizable as such within the zooecium, having tentacles and other organs well formed (fig. 10). It is not yet contractile and it does not feed, the organs being functionally immature. The zooecium is assuming its characteristic shape and attaining almost adult size. At the end of the first day, a large individual measures nearly 1 mm. in length and 0.17 mm. in greatest width. There is variation in length, however, from 0.7 mm. to 1 mm. and but little variation in width. During the second day the polypide becomes active, extends its tentacles, and feeds. The characteristic spines at the upper margin of the zooecium have also developed (fig. 11)—not seven or nine in number, as described by Barrois and Nitsche for the European B. flabellata, but always five. The young zooecium now measures from 0.9 mm. to 1.2 mm. in length and 0.17 mm. to 0.18 mm. in width. Only the first member of a colony reaches this considerable length. Those which follow have the same width as the first, but only about half its length, or approximately 0.6 mm. The first individual forms the anchor for the colony and differs from all others on this account. The colony may later acquire secondary anchorage by root hairs (Wurzelfäden) or stolons, but not at this early stage of development.

Before the end of the second day, the second individual of the colony begins to form as a bud at the outer posterior margin of the zooecium of the first individual, and is completed during the fourth day (figs. B, C, and D, p. 374).

By studying older colonies it is seen that the first zooecium frequently buds only once, while the second and all succeeding ones bud twice. The figures of young colonies (figs. 12 and 13) show the general position of the buds. Barrois ('77) describes it as dichotomous budding, although it is not quite symmetrical. By the time the fourth series of zooids is formed, the colony has become complicated and branched. At the end of the first week it consists of eight individuals (fig. 13). At the end of ten days twenty or more complete individuals and numerous buds are present. It may be noted that it requires almost two days for a bud to arise and complete its development into a functional zooid.



Figs. B, C, D Camera-lucida drawings to show the origin and development of buds. Figures B and C, forty-eight hours old, showing the upper half of the first zooecium with its first bud forming. Figure D, the same individual ten hours later. Figures B and C contain one complete polypide each. Figure D has also the second polypide sufficiently developed to show tentacles and digestive tract clearly, but they are not represented in the drawings. The development of the characteristic spines is also shown.

The colony of Bugula flabellata soon assumes a radiate or cup-shaped form, due to the mode of branching. It has assumed this form within two weeks and consists of from 100 to 170 individuals (table 3). In fifteen days its breadth exceeds its height, measuring ¼ inch in height and ⅓ inch in diameter, the colony now being circular or urn-shaped. In one month a colony measures ¾ inch or more in diameter and in six weeks, well over an inch. Such colonies consist of thousands of individuals.

The attached end of the colony remains slender. The holdfast of the elongated primary individual remains the only support of the colony until a late period of development (two or three weeks). After the colony consists of 200 or more individuals, slender thread-like outgrowths resembling root hairs (Wurzelfäden of Nitsche) extend from two to several basal individuals and give secondary anchorage to the colony. These Wurzelfäden adhere closely to the primary zooecium, but spread out over the substratum, forming a broad disk-like support.

Avicularia are not formed by the first two individuals of the colony, but they may develop upon all later ones. These curious structures have a striking resemblance to bird heads and have been interpreted to be highly modified zooecia. However, Barrois opposes this view. The first avicularia usually appear on colonies of ten individuals and they are abundant and actively functioning thereafter.

Table 3 gives the relative measurements of the colonies at various ages and shows graphically the rate of growth. The work done during 1924 was repeated in 1925 and 1926, with close agreement in the results. The growth rate as given in the table was fully verified. Specimens of various ages were preserved in vials and when arranged in sequence they give a visual demonstration of rapid growth. The colonies are full grown in two or two and a half months and measure ¾ to ⅞ inch in height and 1½ to 1¾ inches in diameter.

The measurements given in table 3 are not averages, but actual measurements of the largest individuals of the specified ages. It will be noted that not all colonies of the same age have the same measurements. Those which grew in midsummer are usually somewhat larger than those of the same age which grew earlier or later. Numerous examples are included, to give not only a fair conception of the rate of growth, but also the variations due mainly to temperature differences.

Age at sexual maturity and duration of life

A colony of Bugula flabellata one month old expelled a few larvae on July 8th and colonies six weeks old liberated larvae abundantly. Although the colonies continue to grow for two or three months, they come to sexual maturity when they measure approximately ½ inch in length and from ½ to ¾ inch in diameter. They seem to reach their greatest reproductive activity within six weeks. The older and larger colonies proved to be less prolific, and they are senescent at three months. The largest colonies do not survive the winter and therefore die within the year.

Winter condition, encystment, and asexual reproduction

Colonies of Bugula flabellata continue to feed and grow as well as reproduce at Woods Hole until about the middle of November. After this time the polypides are represented by an oval greenish yellow or brown cyst. Each zooecium contains a single cyst-like body of this description and thus they pass the winter. In case they survive the winter, the feeding polypides are reformed between the first and fifteenth of May. The storms of winter leave the colonies lacerated and broken. The older colonies especially are torn to shreds and such parts as are left do not revive. As was stated above, it frequently happens that only the youngest colonies successfully survive the winter period of inactivity.[10]

It was clearly shown that asexual reproduction, especially in May, may take place in a variety of ways. Part of a colony may revive and part remain inactive. One surviving zooecium may start a new colony. The two, three, or several basal individuals of a colony may send out rhizome-like runners or stolons to re-anchor the colony. These stolons may send up buds which develop into new colonies. This varied budding is not in evidence in Bugula flabellata except in the spring, as the surviving colonies revive from the winter hibernation. Stolon formation is common in some species (Calvét, '00). Nitsche figures them in a young colony of Bugula flabellata. I have observed their formation, especially in early spring, in winter-torn colonies as above described. They are purposely called stolons here because new colonies were seen to arise from them. They are, of course, a secondary means by which an uprooted colony may re-anchor itself, and as explained earlier, they form the normal anchorage or supplementary anchorage of all but young colonies.

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[10] According to published accounts, these brown bodies are not true cysts and do not give rise to new polypides, but they have every appearance of being alive.

SUMMARY

The breeding season of Bugula flabellata extends from June 10th to November 15th. The young embryos develop in brood pouches (ovicells) and are finally expelled from the colony as swimming embryos. They come from the colonies at dawn or early morning. After a free-swimming period of four to six hours, each larva becomes attached and after a profound metamorphosis which involves the loss of larval organs it develops into the bryozoan colony by budding.

The larvae at first are positive to light, but become negative before attachment. This reversal of behavior enables them to scatter widely and also to find a suitable support. Their behavior is described in detail and the mode of attachment is explained.

After the larva has become attached, a period of rapid growth by budding ensues. The rate of growth is given in a table in which it is shown that the first individual of the colony is completed in two days and that a new series of buds is formed every two days. There are eight or more individuals in one week and over a hundred in two weeks. In one month the colony is half grown and becomes sexually mature. A colony becomes senescent in three months, when it measures 1½ to 1¾ inches in diameter.

The polypides hibernate as cysts or brown bodies and resume growth in early May when new polypides are formed. The chief contribution of the paper is a study of the behavior of the larva, its mode of attachment, the rate of growth of the colony, and age at sexual maturity. The life history and duration of life are given.

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BIBLIOGRAPHY

BARROIS, J. 1877 Recherches sur l'embryologie des Bryozoaires. Monograph, Travaux de l'Inst. Zool. de Lillie.

——— 1879 Mémoire sur la métamorphose des Bryozoaires. Annales des Sciences Naturelle, T. 9.

——— 1882 Embryogénie des Bryozoaires. Jour. d'Anat. et Physiol., T. 18, p. 1.

VAN BENEDEN, J. 1845 Recherches sur l'anatomie, la physiologie et l'embryogénie des Bryozoaires. Bruxelles, Mém. de l'acad. Belg., T. 18–19.

BORG, F. 1923 On the structure of cyclostomatous Bryozoa. Arkiv. für Zoologie, Bd. 15, S. 1.

——— 1926 On the body wall in Bryozoa. Quart. Jour. Micr. Sci., vol. 70, p. 584.

CALVÉT, L. 1900 Contribution à l'histoire naturelle des Bryozoaires ectoproctes marins. Thesis pres. à la Faculté des Sciences de Paris, Trav. Inst. de Zool., NS. 8.

DAVENPORT, C. B. 1891 Observations on budding in Paludicella and some other Bryozoa. Bull. Mus. Comp. Zoölogy, Harvard College, vol. 22, p. 1.

——— 1892 Germ layers in Bryozoan buds. Zool. Anz., Bd. 15, S. 261.

HARMER, S. F. 1897 On the development of Lichenopora verrucaria. Quart. Jour. Micr. Sci., N.S., vol. 39, p. 71.

——— 1887 Sur embryogénie des Bryozoaires. Archiv. de Zool. Exp., series 2, T. 5, p. 443.

——— 1903 On the morphology of the Cheilostomata. Quart. Jour. Micr. Sci., vol. 46.

——— 1910 The Cambridge Natural History, vol. 2, p. 465.

HUXLEY, T. H. 1856 A note on the reproductive organs of the Cheilostome Polyzoa. Quart. Jour. Micr. Sci., p. 191.

JOLLET, L. M. 1877 L'histoire naturelle des Bryozoaires des côtes de France. Archiv. de Zoologie Exp., T. 6, p. 193.

KUPELWIESER 1905 Untersuchungen über den feineren Bau und die Metamorphose des Cyphonautes. Zoologica, Bd. 19.

MARCUS, E. 1921 Über die Verbreitung der Meeresbryozoen. Zool. Anz., Bd. 53.

NITSCHE, H. 1870 Beiträge zur Kenntniss der Bryozoen. Zeitsch. für wiss. Zool., Bd. 20, S. 1.

——— 1871 Über die Anatomie und Entwicklungsgeschichte von Flustra membranacea. Zeitsch. für wiss. Zool., Bd. 21, S. 416.

O'DONOGHUE, C. H. 1926 Observations on the early development of Membranipora villosa. Contributions to Canadian Biology, vol. 3.

PROUHO, H. 1890 Recherches sur la larva de la Frustrella hispida. Archiv. de Zool. Exp. et Gén., T. 18, p. 409.

——— 1892 L'histoire des Bryozoaires. Archiv. de Zoologie Exp., 2d series, T. 10, p. 559.

ROBERTSON, ALICE 1903 Embryology and embryonic fission in the genus Crisia. Univ. of Cal. Publ. Zoöl., vol. 1, p. 115.

SCHNEIDER, A. 1868 Über die Entwicklung des Cyphonautes compressus (Ehrenberg). Sitzungsbericht der Gesellschaft Naturforschender Freunde zu Berlin, S. 24.

SEELIGER, O. 1906 Über die Larven und Verwandschaftsbeziehungen der Polyzoen. Zeitschr. für wiss. Zool., Bd. 84, S. 1.

——— 1889 Die ungeschlechtliche Vermehrung der entoprocten Bryozoen. Zeitsch. für wiss. Zool., Bd. 49, S. 169.

WATERS, A. W. 1889 On some ovicells of cyclostomatous Bryozoa. Jour. Linn. Soc. Zoöl., vol. 20.

ABBREVIATIONS

a.o., apical organ
i.s., internal sac
l., lips of the lateral groove
l.g., lateral groove
p.e., pigmented eyespots
p.d., posterior depression or oral depression
r.c., circlet of rigid cilia
v.p., vibratile plume

PLATE 1

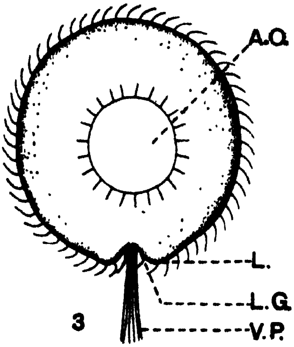
EXPLANATION OF FIGURES

1 and 2 Lateral views of the larva of Bugula flabellata, one showing the lateral groove, *l.g.*, and the vibratile plume, *v.p.*, the other showing the opposite side of the larva which is without lateral depression.

3 and 4 Polar views of the larva of B. flabellata, showing lateral groove, vibratile plume, and apical organ. Figure 4 shows the extent to which the lips of the lateral groove may be protruded or withdrawn by muscular contraction. The dotted line indicates their position when fully contracted.

5 and 6 Side and polar views of the larva of Bugula turrita. It differs from the other only in having pigmented eyespots, *p.e.* Figure 6 shows the relative position of the four eyespots.

7 Posterior view of the larva of B. turrita shown as a semitransparent object. Posterior depression, *p.d.*, the internal sac, *i.s.*, and the lateral groove, *l.g.* The apical organ and two eyespots are shown in dotted lines as seen indistinctly through the side of the larva.



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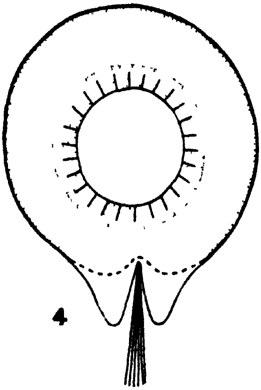

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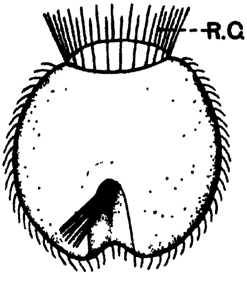

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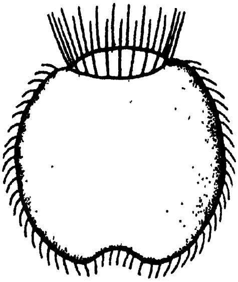

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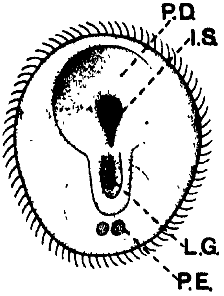

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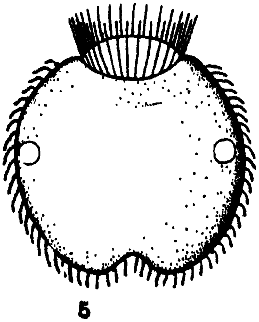

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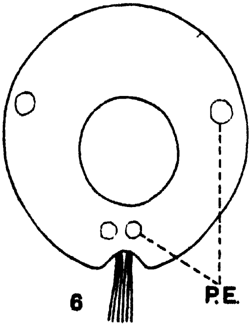

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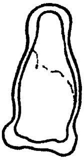
PLATE 2

EXPLANATION OF FIGURES

Camera-lucida drawings to show the rate of development and mode of budding of the colony.

8 Size, 0.23 × 0.15 mm. Four hours after attachment.
9 Size, 0.25 × 0.14 mm. Eight hours after attachment.
10 Size, 0.8 × 0.17 mm. Twenty-four hours after attachment.
11 Size, 1.1 × 0.18 mm. Forty-eight hours after attachment.
12 Size, 2.2 × 0.17 mm. Five and one-half days after attachment.
13 Size, 3.3 × 1.1 mm. Seven days after attachment.



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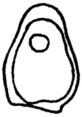

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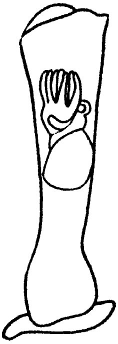

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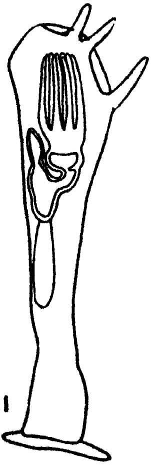

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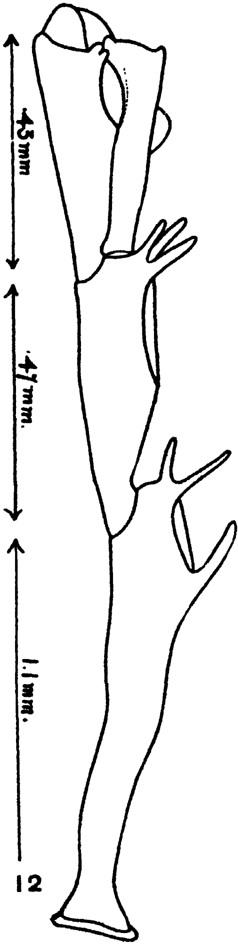

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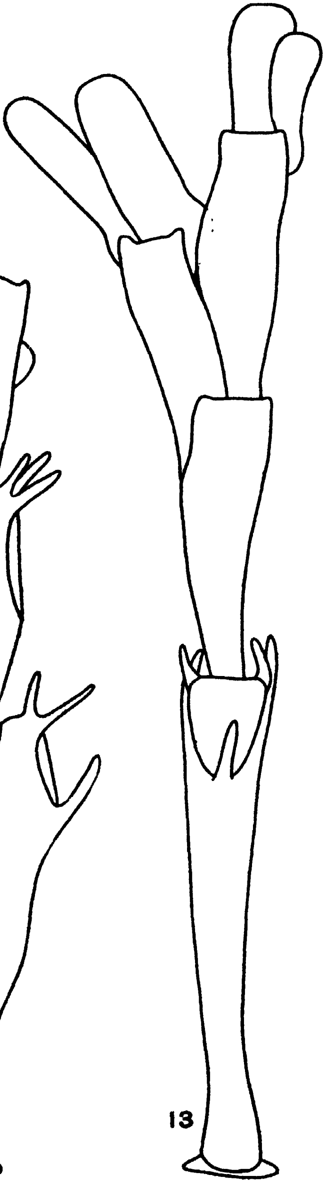

13

THE HISTOLOGY OF THE BLOOD OF SOME BERMUDA ASCIDIANS[1]

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FIVE COLORED PLATES (SIXTY-SIX FIGURES)

AUTHOR'S ABSTRACT

The structure of the living blood cells is described and the cells are classified in the following species of ascidians: Phallusia nigra, Ecteinascidia turbinata, Clavelina oblongata, Symplegma viride. Evidence for the genetic relationship of the various types of cells is discussed.

During the summer of 1926, I made a study of the blood of several species of ascidians. The work was carried on at the Bermuda Biological Station for Research. I wish to express my thanks to Prof. E. L. Mark for the privilege of using the facilities of the station and for his kindness and assistance. I am indebted also to Dr. Willard G. Van Name for identifying some of the species for me. I have used his 1921 paper(1) in identifying the other species.

In a previous paper(2) I gave a description of the types of cells found in the blood of Perophora viridis and undertook to group the types of cells recorded in the literature for other ascidians with those found in P. viridis. Because of the great variation that undoubtedly exists in the blood of different ascidians and the incompleteness of the recorded descriptions, it was not possible to group with assurance on the basis of homology the varieties of cells reported in different species. On account of the interest in and biological importance attaching to the types of cells found and in order to investigate further the matter of their homologies, it has seemed worth while to continue the study of ascidian blood.

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[1] Contributions from the Bermuda Biological Station for Research, no. 157.

As in my previous study, the methods here used consisted in examining the living blood both unstained and stained with vital dyes and in treating the cells with various reagents while under observation. Also fixed and sectioned material has been studied. This, however, did not add anything of importance to the observations made upon fresh blood. Fixation brings about such profound changes in the cells that it is scarcely possible to recognize in fixed material some of the cell types found in the living blood.

The description of the blood cells of the different species I have studied follows.

PHALLUSIA NIGRA SAVIGNY (ASCIDIA ATRA LESUEUR)

1. Green cells (figs. 1, 2, 3). Large rounded green masses virtually fill the cell body, save for a region occupied by the nucleus. Except for a slight difference in color, these cells are approximately like those I have previously described for P. viridis. The green masses blacken readily with osmic acid; they may be caused to disappear by running a little 1 per cent acetic acid under the cover-glass. There is then left a cell with a nucleus, cytoplasmic reticulum, and vacuoles where the green bodies were located.

The green bodies of these cells evidently consist of the fluid contents of vacuoles and they have a somewhat highly refracting surface of fatty nature and green color. The refractive quality of this surface film makes the green bodies opaque. The addition of very weak acetic acid (about 1 to 500), not strong enough to disrupt the surface membrane, increases the refractiveness and opacity. Weak ammonia decreases it. I add a drop or two of weak ammonia (about 1 to 200) to the edge of a cover-glass preparation of blood. Without changing the color of the green globules, it reduced the refractiveness of the surface films and caused them to become transparent. In numerous cells oscillating granules, some of them coalesced into larger masses, were revealed in the interior of the green globules (fig. 3). This suggests that these green cells are derived from vacuolated compartment cells with dancing granules.

The green cells at times are noticeably amoeboid. I have observed the formation and movement of long, clear, filamentous pseudopodia and have seen the green bodies flow into them, thus forming large pseudopodia.

2. Orange cells (fig. 4) have a clear region free from granules, in which the nucleus can be demonstrated with acidulated methyl green, and globular orange-colored masses crowding the cell body. These colored masses do not differ greatly in size and shape from the green bodies of the green cells, but do differ chemically, as is shown by the fact that they do not blacken with osmic acid. They appear to be finely granular. The orange cells, too, are amoeboid.

3. Colorless morula cells (figs. 5, 6, 7). The morula cells commonly appear to be composed of three vacuolated lobes and a peripheral cap-like nuclear region. Figure 5 represents one of these cells drawn from life. While the cell is alive the vacuolated lobes are virtually opaque. Figures 6 and 7 were drawn from cells after fixation. Osmic acid does not blacken them. The surface films (or perhaps the entire contents of the vacuoles) stain with methylene blue. These cells are quite abundant.

4. Coarsely granular amoebocytes (fig. 8). There is a clear peripheral layer of hyaloplasm and a coarsely granular endoplasm. As in other species of ascidians, cells of this type were seen occasionally with ingested cells of other types, showing them to be phagocytes.

5. Amoeboid compartment cells (figs. 9 and 10) appear to be hyaline while alive and unstained. When weak neutral red in sea-water is added to the edge of the cover-glass, the cells after about half an hour begin to show evidences of being reticulated and of containing angular vacuoles. Small dancing stained granules appear in the fluid of the vacuoles. Some of the granules may be observed to coalesce. These cells are conspicuously amoeboid and form both blunt and long slender pseudopodia.

6. Vesicular signet-ring type of cell (figs. 12 and 13). There is in these cells a peripheral thin layer of cytoplasm, which

is thicker in the region where the nucleus is located, and a single large vacuole filled with a clear colorless fluid, which may or may not contain brownian granules stainable with methylene blue, neutral red, and other dyes. After treatment with osmic acid, the fluid turns gray and the granules blacken. The grayness of the fluid is due most likely to the presence of ultramicroscopic particles of a fatty nature. These cells were seen to be amoeboid at times. Figure 11 represents a similar cell with two vacuoles. Figures 9 to 12 form a series representing what are probably transition stages from the compartment cell to the vesicular type.

7. Blue cells (figs. 15, 16, and 17). These cells consist of clear colorless protoplasm in which globular blue masses of varying size are embedded. The blue masses or granules vary in color from gray-blue to deep blue-black. There is a region of the cell free from granules and in this is located the nucleus, which can be demonstrated by staining with acidulated methyl green. Weak acid dissolves or decolorizes the blue bodies, as it does the green bodies of the green cells. There is then left a cell with reticular protoplasm enclosing large vacuoles very similar in appearance to those of the colorless compartment cells. The blue cells in the blood are commonly globular (fig. 15), but in cover-glass preparations I have observed them moving along by amoeboid activity. Figure 16 represents a cell with slender tapering pseudopodia. Figure 17 was drawn from a cell showing an interesting physiological phase in which long slender flagella-like processes had been formed and were moving about. The processes had very small granules (apparently blue) at intervals along their length and a somewhat larger granule at the tip of each. This cell is figured from a cover-glass preparation of blood in sea-water that had been standing for three and one-half hours. What factors caused the formation of these processes I cannot say. It may have been lowered oxygen concentration in the water or increased salt concentration resulting from evaporation. Many cells were like this.

Blue cells are found not only in the blood, but also in the test. They are more abundant toward the surface of the test than in deeper parts. It is to them that Phallusia nigra owes its deep blue color. In addition to the blue cells, the test contains large 'bladder cells,' similar in appearance to the signet-ring cell, but with a diameter about five times as great.

8. Finely granular amoebocytes (fig. 14). Cells of this type resemble mammalian lymphocytes. They show a peripheral zone of finely granular cytoplasm surrounding a fairly large nucleus with scattered chromatin masses. These cells are not easily found in unstained blood. By staining fresh blood with eosin in sea-water one can find a few such cells.

So far as I know, only two papers dealing with the blood of this species have appeared hitherto. The earlier one, by Hecht(3), is primarily physiological in nature, and since cytological observations in it are very meager, we need not consider it here. The later paper, by Fulton(4), gives an account of the several types of cells found by him in the blood. Since some of his observations and conclusions differ from mine, it seems necessary to discuss these differences.

In considering the green cells Fulton speaks of a cytoplasmic center around which the green granules are located, and remarks that "The exact nature of the so-called cytoplasmic center was difficult to determine." I have shown that this cytoplasmic center of the cell encloses the nucleus. He did not observe amoeboid activity in these cells nor in the orange cells. I can state with certainty that both types of cells are amoeboid. He speaks of a rapid swimming movement of the orange cells; but I failed to observe it. Fulton states that the blue cells differ from other types of pigmented corpuscles in the possession of a contractile vacuole. Also he attributes contractile vacuoles to what he calls A-2 cells, which appear to be the same as those that I have described as coarsely granular amoebocytes. I am convinced that no contractile vacuoles are present in these cells. The presence of the clear region occupied by the nucleus may have led him into error. The movements of the cell might at times have given to this region an appearance simulating that of a contractile vacuole. I failed to find the 'brown cell' described by Fulton, which he says is seldom met with. It is possible that brown cells similar to those to be described further on for Clavelina oblongata and Symplegma viride may be found occasionally in the blood of Phallusia nigra. Fulton describes three types of unpigmented actively moving cells, which he designates as A-1, A-2, A-3. His A-1 cells, he says, are amoeboid and perfectly homogeneous. I believe that these cells are probably the same as my amoeboid compartment cells, a type which he did not describe and which appear to be homogeneous unless closely examined. The lymphocyte type of cell he also failed to describe. Perhaps these, too, should be included under his group of homogeneous (A-1) cells. His highly granular (A-2) cells are obviously the same as my coarsely granular amoebocytes. His belief that this cell contains a small contractile vacuole has been referred to previously. Fulton describes four types of unpigmented quiescent cells (Q-1, Q-2, Q-3, and Q-4). The Q-1 and Q-2 cells are obviously the same as those that I have classified under the heading of vesicular signet-ring type of cells. Contrary to Fulton's belief, these cells are at times amoeboid. His Q-3 and Q-4 cells (bilobed and trilobed) are the same as those that I have classified as colorless morula cells.

Fulton proposed a theory of the origin of the green cells that does not seem tenable in the light of our present knowledge of the structure of ascidian blood cells. He reports having caused the vesicular signet-ring type of cell (his Q-2 cell) to metamorphose, under the influence of weak organic acids, into green cells. I applied acetic acid (one of the acids used by him) in varying concentrations to check his observations. The acid does appear to bring out a faint green color in various colorless cells; but I did not observe it to cause the transformation of any cell into a green cell. Apparently what happens is as follows: weak acids of the proper concentration kill the cells without distorting them and reveal certain details of structure not ordinarily visible in the unstained living cell. For example, it reveals the vacuolar structure of the compartment cells, which when alive and unstained appear to be hyaline amoebocytes. There can be picked out, then, in a microscopic field a series of cells that might be considered to be transformation stages between the vesicular type of cell and the green cell. The greenish color that seems to be produced in many cells is not, I think, a pigment, but a refraction phenomenon resulting from the action of the acid on the surface films of vacuoles. Furthermore, it does not seem probable that a vesicular signet-ring type of cell (or 'bladder cell') could metamorphose by fragmentation or otherwise into a cell having the structure of a green cell. There is nothing to fragment except the fluid and brownian granules present in the vesicle. If one were to grant that the fluid contents of the vesicle became a gel and fragmented (for which I observed no evidence), I do not see how septa of cytoplasm such as are present between the green bodies of green cells could be formed between the fragments.

ECTEINASCIDIA TURBINATA HERDMAN

1. Green cells (figs. 18 and 19). Except for the fainter color of the globules, these cells are about like the green cells in Phallusia nigra and Perophora viridis. As in those forms, acid destroys the green globules, leaving a reticulated cell with vacuoles where the green bodies were located and a nucleus in the clear protoplasmic region. Osmic acid blackens the greenish globules. In a cover-glass preparation of blood treated with osmic acid the blackening resulting from the osmic acid soon fades, and, as it does so, the globules are seen to be granular. This suggests a relationship between them and the granular compartment cells. There is a type of colorless amoeboid cell (fig. 22), abundantly present in the blood stream, which may be an intermediate stage between the compartment type of cell (figs. 26, 27, and 28) and the green cell. Similar cells are found within the substance of the test, in addition to the transparent cells with compartments and with dancing granules. These are essentially similar in appear-

ance to the compartment cells, except that the vacuoles are not transparent, the lack of transparency being due apparently to the high refractive quality of the surface films of the vacuoles.

2. Orange cells (figs. 20 and 21). In Ecteinascidia the orange cells are commonly quite large and numerous. The clear cytoplasm of the cell is fairly crowded with spindle-shaped orange bodies, which may vary considerably in size. Simpkins(5) says that they range from 3 to 12 μ in length. I did not make micrometer measurements, but calculations from camera sketches show a variation of 3 to 6 μ. This is the only species found in which the orange bodies were spindle-shaped. There is a region free from them, in which the nucleus may be demonstrated by the use of acidulated methyl green. The orange cells are especially abundant in the mantle around the inhalent and exhalent siphons. They are not abundant in the mantle at the bases of the zooids. These pigment cells in the mantle are commonly seen to be spread out into very thin films (fig. 21), forming a pigmented curtain over the deeper tissues, whereas those circulating in the blood are rounded up into globular shape. Osmic acid has no apparent immediate effect on the orange granules.

Simpkins in an interesting paper (l.c.) dealing with the transmission of pigment from one generation to the next in Ecteinascidia reports the engulfing of fusiform pigment corpuscles by the growing egg—the early oogonium being devoid of color. These orange corpuscles are then dissolved and the pigment absorbed by the accumulating yolk spherules. In the developing egg he has observed the condensation of the orange pigment of the yolk to form fusiform corpuscles in what he calls pigment patches. Simpkins failed to find a nucleus in these pigment patches and so arrived at the conclusion that they were not cells and that they bore no relation to the blood. There can be little doubt but that his 'pigment patches' are in fact identical with the orange cells described above and found in the blood stream or as wandering cells in the tissues.

3. Colorless morula cells (fig. 24). These cells apparently consist of a nucleus and a small amount of dense cytoplasm containing three or more large vacuoles. In the blood of some immature individuals taken from 'Nonsuch' Island cells of this type constituted a very distinct group and were in appearance about like those previously described for P. viridis.

There is another variety of amoeboid cells (fig. 49), not very numerous, which I hesitate to classify. They may be related to the morula cells or they may be a stage in the development of green cells. They contain spherical, sharply defined, colorless vesicles whose surface membranes are highly refractive. When the cell is undergoing amoeboid activity, these spherical vesicles roll over one another without appreciably changing shape, indicating a high degree of turgidity within them. Sometimes cells are seen in which these vesicles darken considerably when treated with osmic acid; usually, however, they do not blacken with osmic acid and they do not stain with sudan III.

4. Coarsely granular amoeboid cells (fig. 23) are quite abundant. The endoplasm is coarsely granular, but the ectoplasm is clear and denser. They are obviously phagocytic. Granular cells from a specimen that had been in the laboratory for several days, in which degenerative changes had apparently been going on, showed orange granules and other obviously ingested granular masses in the endoplasm. Some of the ingested spindle-shaped bodies from orange cells were without orange color, presumably having lost it through digestive changes. Some of the larger endoplasmic masses sometimes blacken with osmic acid and look as if they might be ingested globules of green cells. These amoeboid cells are very active, and they, too, sometimes spread out into broad thin sheets.

5. Amoeboid compartment cells (figs. 26, 27, and 28). These are colorless cells and have a variable number of large, somewhat angular vacuoles, which usually show dancing granules in the vacuolar fluid. The granules are ordinarily invisible

when unstained. They stain a deep blue with Janus green B, and they also take neutral red and methylene blue. Here, as previously noted for P. viridis, in some cells the granules blacken with osmic acid and in some they do not. This type of cell is present in the test as well as in the blood vessels.

6. Signet-ring type of cell (fig. 25). These cells are quite like the corresponding cells described here for Phallusia nigra and in my previous paper for Perophora viridis. Sometimes there are no distinguishable granules in the vacuolar fluid; at other times, a few; at still others, many, some of which may be observed to coalesce into larger masses. The granules blacken with osmic acid. Occasionally, cells with two or three vacuoles (fig. 26), probably transition stages from compartment cells, are to be found.

7. Finely granular amoeboid cells (figs. 29 and 30). These are small cells of the lymphocyte type. When stained, they show a nucleus containing a fair amount of chromatin enveloped in finely granular cytoplasm. This is probably a primitive type of cell.

CLAVELINA OBLONGATA HERDMAN

1. Green cells (fig. 31) of this species have clear transparent cytoplasm in which are embedded sharply outlined large bodies and smaller spherules, both having a higher index of refraction than the protoplasm in which they are enveloped. They have a very pale yellowish green color—so pale that it is difficult to decide whether the color is due to pigment or merely to a refraction phenomenon. With proper illumination some of the larger masses may be seen to be made up of smaller less well-defined bodies, as indicated in figure 31, the line of separation between the smaller components being barely distinguishable. There is a region of protoplasm devoid of visible spherules or masses, in which a nucleus may be demonstrated by the use of acidulated methyl green. Under the influence of the acid the greenish bodies break up and dancing granules that take the stain become visible. Soon these also disappear, leaving a reticu-

lated cell. Sometimes the green bodies vanish without the appearance of any granules. These cells differ from the green cells described for other species in that the green masses do not blacken with osmic acid. The surface film, therefore, is probably albuminous and not fatty. Green cells (figs. 32 and 33) are found in the test as well as in the blood of this species.

Amoeboid cells with colorless spherules (fig. 34), which I look upon as young stages of the green cells, are also found in test and blood. Superficially, they look more like the 'coarsely granular amoebocytes,' but weak acid destroys the spherules in these cells, as it does in the green cells, whereas it does not destroy them in the coarsely granular amoebocytes.

It is of interest to note that the Bermuda Perophora viridis, as I have observed elsewhere (p. 399), presents, in both test and blood, cells that I interpret as young stages of green cells. In Perophora the young stages, as well as the mature green cells, blacken with osmic acid, whereas in Clavelina neither the one nor the other does.

No orange cells were found in this species nor were colorless morula cells found, but there is present a brown cell that I consider to be the probable homolog of the morula type.

2. Brown cells (figs. 35, 36, and 37). These cells usually have three or more vacuoles filled with a clear fluid which is crowded with small brown dancing granules. Osmic acid darkens these granules considerably. There is a clear region in the cell which contains the nucleus. A good many of these cells were seen engulfed in granular amoeboid phagocytes.

3. Coarsely granular amoebocytes (fig. 38). These cells exhibit a clear ectoplasm without granules and a more fluid, coarsely granular endoplasm. A nucleus may be demonstrated with acidulated methyl green. These cells seem to be more numerous in material kept under unfavorable laboratory conditions, and consequently undergoing degenerative changes, than in fresh material. They are phagocytic. I have seen them with ingested purple and yellow granules and with ingested purple and brownish cells.

2. Orange cells (figs. 52 and 60) contain numerous globular reddish orange bodies. They are present in the blood and are fairly abundant in the mantle around the branchial and atrial siphons; they are also aggregated to form reddish orange streaks and splotches in the mantle lower down on the body. There was a noticeable difference in the size of these granular bodies in the orange cells from the two colonies. (Compare figs. 52 and 60.)

3. Brownish cells (figs. 54, 55, 56, and 62) contain from one to several large vacuoles filled with a clear colorless fluid containing numerous yellowish brown or greenish brown granules showing characteristic brownian movement. The granules did not blacken with osmic acid. I believe that it is probably correct to look upon these cells as colored variations of the compartment and signet-ring types of cells.

4. Coarsely granular amoeboid cells containing other ingested cells were identified in the blood spaces in sectioned and stained material. No notable difference was observed between these cells and cells similarly classified in the preceding species.

5. Amoeboid compartment cells (figs. 53 and 63) similar to the colorless compartment cells of other species were observed in the test. Dancing granules were found in the vacuoles of some cells. In my hurried examination of this species I did not see these cells in the blood, but I believe that a more thorough study would have shown them to be present.

6. Large greenish blue cells (fig. 61) containing several large vacuoles were found. The contents of the vacuoles make them turgid and they are rendered angular by mutual pressure. The coloring matter of the cells is contained within the vacuoles. Weak acids quickly remove the color and the presence of small dancing granules is revealed. Osmic acid does not blacken, but decolorizes the contents of the vacuoles. Now and then cells of this structure, but of a pale salmon-pink color, were found. These cells were observed only in the blood of the colony with brownish zooids. I am uncertain whether to look upon them as homologs of the colorless

morula cells of other forms or as variations of the compartment cells.

7. Small, finely granular amoeboid cells (figs. 64, 65, and 66) of the lymphocyte type were found in sectioned and stained material of both colonies. In fresh blood from the colony of whitish zooids I found cells (figs. 57 and 58) that I look upon as variations, developmental stages, from this type of cell. There is a fairly broad peripheral layer of hyaloplasm surrounding finely granular endoplasm. In some cases the endoplasm consists of a single mass (fig. 58), in others it is divided into two or more masses (fig. 57). It is conceivable that these cells are stages in the development of primitive lymphocyte cells into vacuolated cells. The transformation of a cell of this type into a vacuolated cell might occur through the conversion of the endoplasmic substance from a gel or semisol state into a sol state. The ectoplasm would remain as the vacuolar envelope.

It may be of some value to record that in still another species (unidentified) I found green cells with reddish orange granules scattered among the green globules. The green globules blackened with osmic acid, but the orange globules did not. There were also typical orange cells and large amoeboid cells with dancing granules. Since I did not find sufficient material, I was not able to make a study of all types of cells in this species.

In addition to these new forms studied, I made a brief examination of some specimens of the Bermuda Perophora viridis to see if the blood presented any marked variations from that of the Beaufort form. Besides the previously described types of cells, I found variations of the amoeboid compartment cell in the blood and in the test. In these the vacuoles had colorless refractive vacuolar membranes that rendered them opaque. Sometimes cells were found having several vacuoles with refractive membranes and one or more without a refractive membrane, and therefore transparent (figs. 47 and 48). The transparent vacuoles showed granules with brownian movement. I look upon these cells as intermediate stages between compartment and green cells.

After finding the finely granular lymphocyte type of cell in other species, I made a special examination of Perophora blood and found the same type present there also. In my previous study I failed to recognize it as a blood cell; but upon going over my sectioned and stained material of the Beaufort Perophora, I found the lymphocyte type of cell in the blood spaces.

COMPARISON AND DISCUSSION

We do not yet know enough about the functions of ascidian blood cells to justify an attempt at classification of them on the basis of physiology; but a morphological grouping might be of some value. Upon consideration of the species that I have studied, it seems that the most obvious classification on the basis of morphology is into vacuolated and non-vacuolated cells. In the group of the non-vacuolated cells we should place the finely granular amoebocytes (lymphocyte type), the coarsely granular amoebocytes, and the orange cells. That these cells at times may contain vacuoles is most probable; but the presence of vacuoles is not an obvious feature of their structure. The grouping of them together is merely for convenience and should not convey the idea that they are more closely related to one another than to other types of cells. Under the heading of vacuolated cells I should place all the other ascidian blood cells that I have studied: the various modifications of the green-cell type, the colorless compartment cells, the morula type of cell, the signet-ring type of cell of different species, the brownish vacuolar cells of Symplegma viride, and the blue cells of Phallusia nigra. It might appear as if the last should be grouped with the non-vacuolar cells, but in view of the fact that the blue masses are so easily destroyed by weak acids leaving vacuolated cells (in this respect being similar to the green cells), I am disposed to consider the blue granules or masses to be a fluid or semifluid substance filling vacuoles within the cells.

As to the genetic relationship of the different types of cells, the evidence is not conclusive; but it is strongly suggestive.

There is evidence to indicate that some of the most different-appearing cells are closely related. That the green cells and the orange cells have a common progenitor is suggested by the fact that in some species the green cells have orange granules as well as green globules. This is true in Symplegma viride and in an unidentified simple ascidian. It is evident at least that the same protoplasm that produces the greenish globules is capable of producing orange granules also.

That the green cells are closely related to the compartment cells is suggested by the following facts: 1) When the green globules are destroyed by acids, hydrogen peroxide, alkalies, etc., the green cells show a structure similar to compartment cells. 2) When green cells of Phallusia nigra are acted upon by very weak ammonia, the globules become transparent and dancing granules similar to those in the compartment cells are seen. 3) Though compartment cells and signet-ring cells are the only ones found in the test of the Beaufort Perophora viridis, in the Bermuda individuals these cells are found and also others, similar to compartment cells, in which the vacuolar contents have formed surface films of a fatty nature (they blacken with osmic acid) similar to the surface film of green globules. 4) In Clavelina oblongata, when the green cells are acted upon by weak acid resulting in the destruction of the globules, or globule films, brownian granules are seen indicating a structure within the vacuoles similar to that found in compartment cells. 5) In Clavelina oblongata there are to be found in the test, *a*) compartment cells, *b*) finely granular cells from which the compartment cells are probably derived, and, *c*) greenish cells, perhaps derived from the compartment cells through the formation, in the vacuoles or their surface films, of albuminous or fatty substance and the acquisition of a small amount of green color.

That the compartment cells and signet-ring cells are closely related to each other seems evident from the fact that transition stages between them may be found. The transformation of a compartment cell into a signet-ring cell could easily come about by the disappearance of the thin partitions between vacuoles. That some of the signet-ring cells contain colored granules in certain species while in other species they contain only colorless granules does not seem to be a matter of great moment, since the color in these seems to be due to the presence of vanadium within the cells (Henze, 7, 8), and vanadium compounds may be colorless or show a great range of colors.

On the basis of what we know about blood cells in other animals and the nature of the primitive cells in general, it seems probable that the finely granular amoebocyte, with its large nucleus and small amount of cytoplasm, is the most primitive type of cell in the blood and the one most closely related to the common-stem cell—assuming for the present a monophyletic origin of the various blood cells.

Cells that may well be transition stages between the lymphocyte type and other types are to be found. It appears probable that the lymphocyte cells through the acquisition of granules may become coarsely granular cells or by the acquisition of vacuolar spaces may be transformed into 'compartment cells.' The colorless morula cells, which are similar in structure to the compartment cells, might have a similar origin. The compartment cells, by the breaking down of the vacuolar partitions, may be transformed into vesicular signet-ring cells, or through the formation of fatty vacuole films and the acquisition of vanadium pigment they may become green cells. Through the formation of different vanadium compounds in the vacuole contents, the blue cells of Phallusia nigra, the brown and mulberry-colored cells of Clavelina oblongata, and the brownish cells and greenish blue cells of Symplegma viride might result from compartment cells. Orange cells probably represent a separate line coming directly from lymphocytes by the formation of orange granules within the cells.

LITERATURE CITED

1 Van Name, Willard G. 1921 Ascidians of the West Indian region and southeastern United States Bull. Amer. Mus Nat Hist, vol 44, pp 283-494.

2 George, W. C 1926 The histology of the blood of Perophora viridis (Ascidian) Jour Morph. and Physiol, vol. 41, pp. 311 331

3 Hecht, S. 1918 The physiology of Ascidia atra Lesueur III The blood system Amer Jour. Physiol., vol 45, pp 157–187 (Contrib. Bermuda Biol. Sta, no. 80)

4 Fulton, John F 1920 The blood of Ascidia atra Lesueur; with special reference to pigmentation and phagocytosis Acta Zoologica, Arg 1, Heft 3, S 381-431 (Contrib Bermuda Biol Sta., no 125)

5 Simpkins, Cleveland S 1924 Origin of the pigment of Ectenascidia Acta Zoologica, Arg 5, Heft 3, S 425 438 (Contrib Bermuda Biol. Sta., no 145)

6 Spek, Joseph 1927 Über die Winterknospenentwicklung, Regeneration und Reduktion bei Clavelina lepadiformis und die Bedeutung besonderer 'Omnipotenter' Zellelemente fur diese Vorgange Arch f. Entwmech Org., Bd 111, S 119 172

7 Henze, M. 1911 Untersuchungen uber das Blut der Ascidien I. Mitteilung Die Vanadiumverbindung der Blutkorperchen Zeits physiol Chem, Bd 72, S 494–501

8 1912 Untersuchungen uber das Blut der Ascidien II. Mitteilung Zeits. physiol Chem, Bd 79, S 215 228

EXPLANATION OF PLATES

All figures are camera-lucida drawings at a magnification of 2400 diameters, except figure 21, which is magnified approximately 850 diameters.

PLATE I

EXPLANATION OF FIGURES

Phallusia nigra

1 and 2 Green cells from a cover-glass preparation of living blood unstained.

3 Green cell unstained, but treated with a 1:200 solution of ammonia.

4 Orange cell from living blood unstained.

5 Colorless morula cell from living blood unstained.

6 and 7 Colorless morula cells, after fixation with osmic acid.

8 Coarsely granular amoebocyte, from living blood unstained.

9 Amoeboid compartment cell, from living blood unstained.

10 and 11 Vacuolated cells after fixation and staining. Probably transition stages between 9 and 12.

12 and 13 Vesicular signet ring cells, from living blood stained with methylene blue.

14 Finely granular amoebocyte, fixed and stained.

15 to 17 Blue cells, living, unstained. The nucleus in figure 15 was drawn after treating the cell with acidulated methyl green.

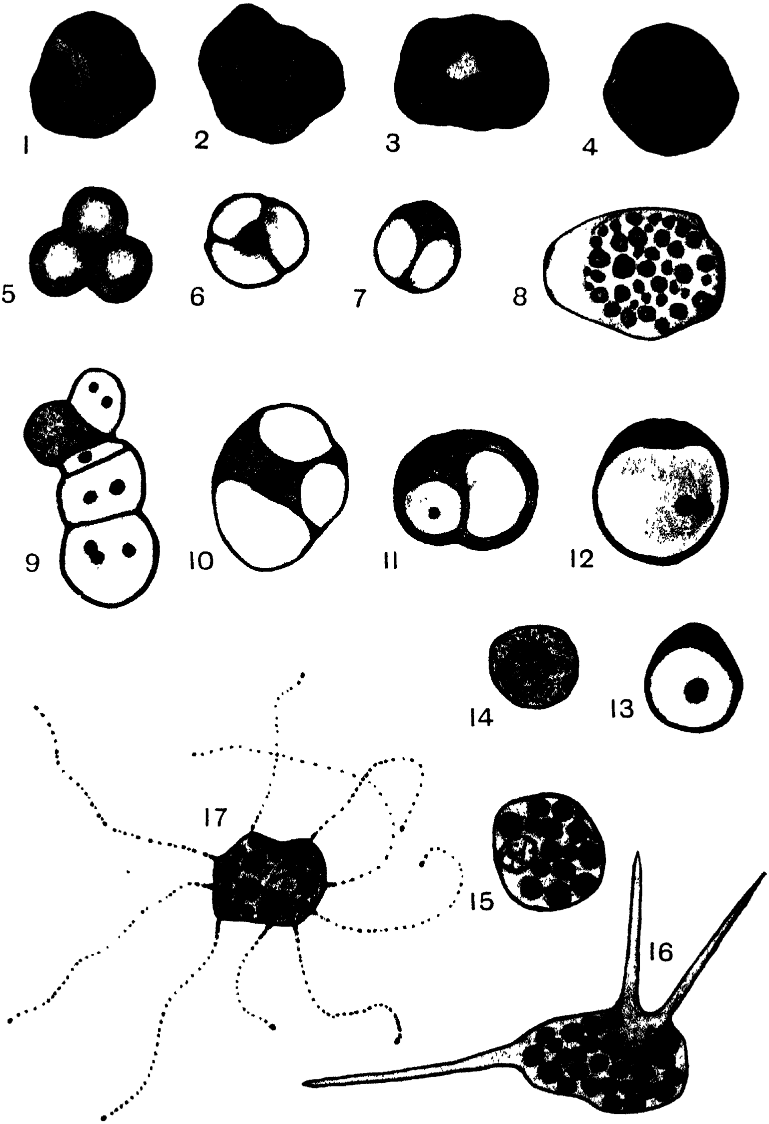
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PLATE 2

EXPLANATION OF FIGURES

Ecteinascidia turbinata

All figures except 29 and 30 are from unstained living blood.

18 and 19 Green cells.

20 Orange cell.

21 Orange cell from the mantle which has spread itself out as a thin film of protoplasm. × 850.

22 Colorless amoeboid cell.

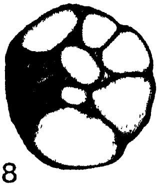
23 Coarsely granular amoeboid cell.

24 Colorless morula cell.

25 Vesicular signet-ring type of cell.

26 to 28 Amoeboid compartment cells.[1]

29 and 30 Finely granular amoeboid cells (lymphocyte type). Drawn after fixation.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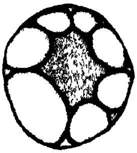
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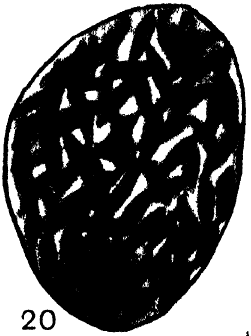
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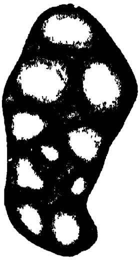
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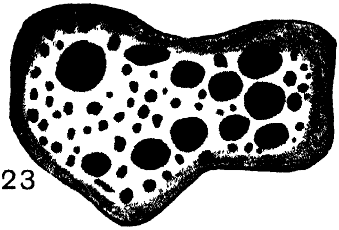
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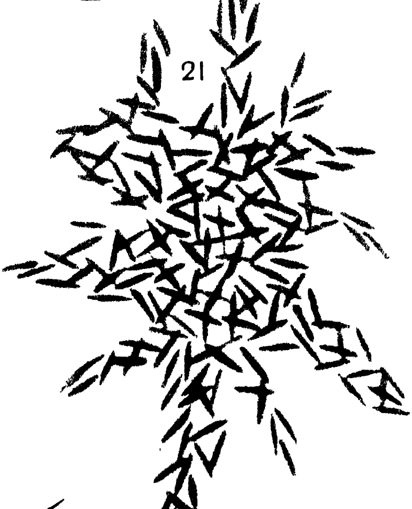
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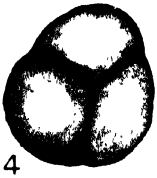
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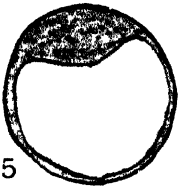
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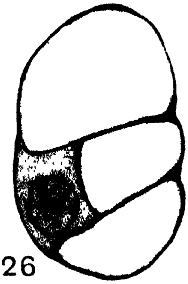
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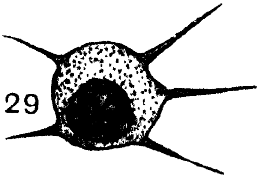
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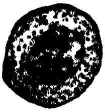
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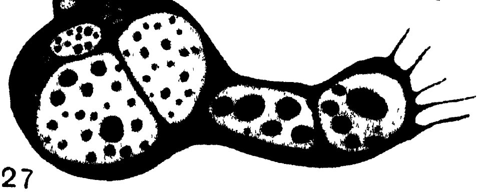
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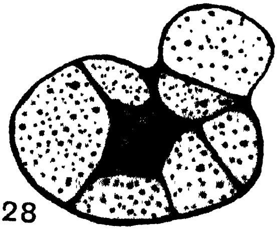
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PLATE 3

EXPLANATION OF FIGURES

Clavelina oblongata

31 to 33 Green cells from test similar to the green cells of the blood. Drawn from living cells unstained.

34 Colorless amoeboid cell from living blood unstained. Nucleus drawn after treatment with acidulated methyl green.

35 to 37 Brown cells from living blood unstained.

PLATE 3

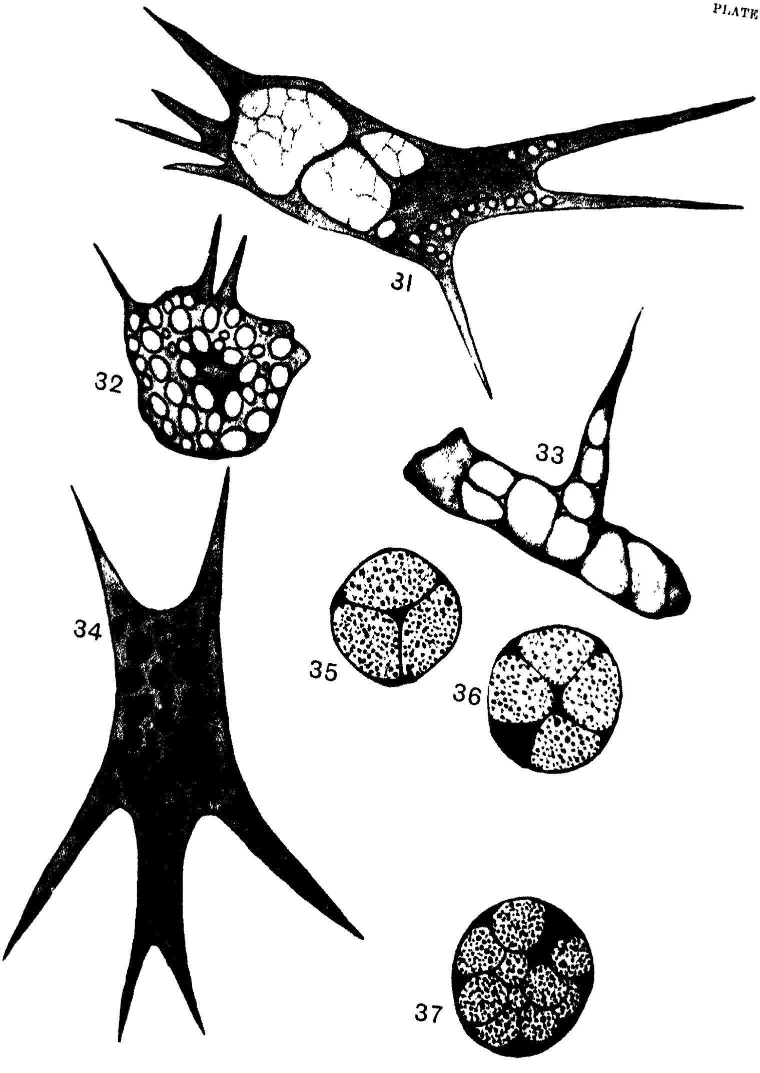


PLATE 4

EXPLANATION OF FIGURES

Clavelina oblongata (figs. 38 to 45), Perophora viridis (figs. 46 to 48), Ecteinascidia turbinata (fig. 49).

38 Coarsely granular amoebocyte from living blood of Clavelina oblongata.

39 Amoeboid compartment cell from the test of Clavelina oblongata, similar to cells present in blood.

40 Vesicular signet-ring type of cell with mulberry-colored granules. From living unstained blood of Clavelina oblongata.

41 Signet-ring type of cell without colored granules. From living unstained blood of Clavelina oblongata.

42 Cell from the test of Clavelina oblongata. Transitional between 39 and 41.

43 Finely granular amoeboid cell from living blood of Clavelina oblongata. Nucleus drawn after treatment with acidulated methyl green.

44 and 45 Cells from test of Clavelina oblongata.

46 Finely granular amoeboid cell (lymphocyte type) from blood of Perophora viridis.

47 and 48 Colorless vacuolated amoeboid cells from living blood of Perophora viridis. Most of the vacuoles had refractive membranes that rendered them opaque. In each of these cells one vacuole lacked an opaque membrane and brownian granules were seen to be present.

49 Amoeboid cell from living blood of Ecteinascidia turbinata.

PLATE 4

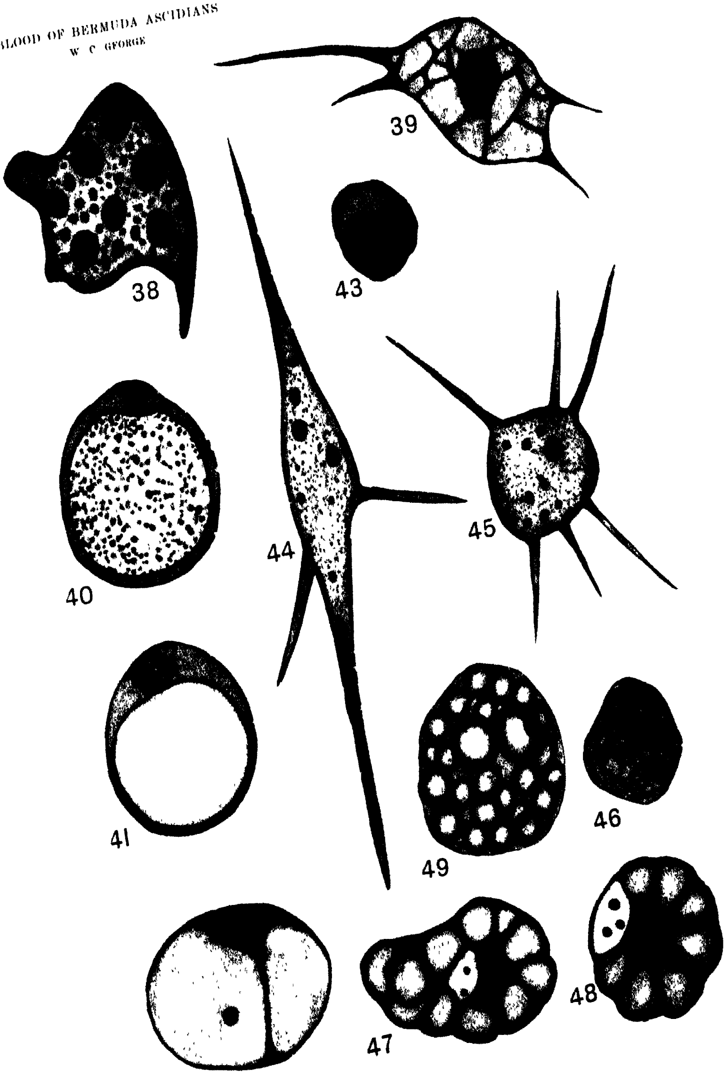


PLATE 5

EXPLANATION OF FIGURES

Symplegma viride

Figures 50 to 52 and 54 to 62 are from unstained living blood.

50 and 51 Green cells from white zooids.

52 Orange cell from white zooid.

53 Compartment cell from test of white zooid.

54 to 56 Yellowish brown cells from white zooids.

57 and 58 Finely granular amoeboid cells from white zooids.

59 Green cell from brown zooid.

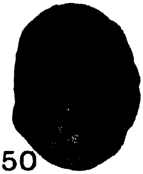
60 Orange cell from brown zooid.

61 Greenish blue cell from brown zooid.

62 Greenish brown cell from brown zooid.

63 Compartment cell from test of brown zooid.

64 to 66 Finely granular amoeboid blood cell (lymphocyte type) from sectioned material.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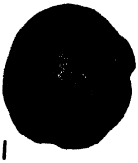
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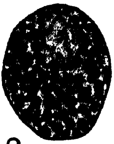
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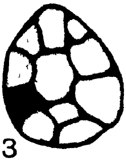
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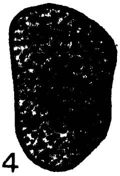
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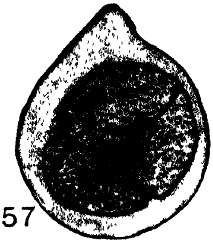
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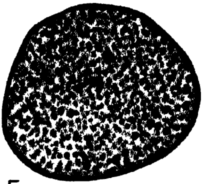
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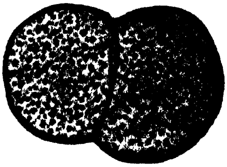
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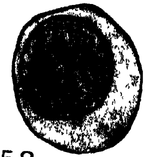
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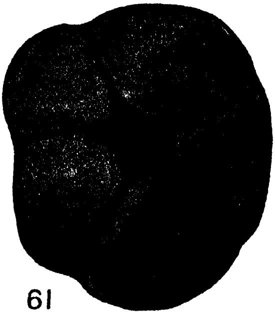
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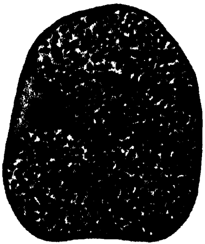
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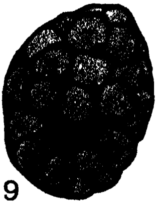
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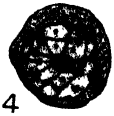
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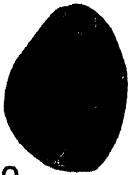
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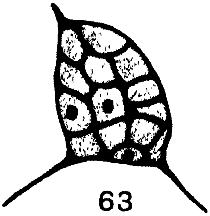
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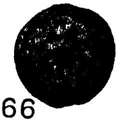
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THE GERM CELLS IN THE HERMAPHRODITIC GLAND OF POLYGYRA APPRESSA

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FOUR TEXT FIGURES AND TWO HELIOTYPE PLATES (TWENTY-SIX FIGURES)

AUTHOR'S ABSTRACT

The male and female germ cells of Polygyra appressa were found occurring together in the acini from the time the snails were 5 mm. in width onward, and their development traced backward to the gonial stage, in which the aspect of the female cells, present in small numbers in the acinus wall, and the male cells, in greater numbers in the lumen, seems identical.

In oocytes, differentiation involves the enormous increase in volume of cell, nucleus, and plasmosome; the appearance of yolk and of a secondary nucleolus attached to the first one, and, in some cells, various other nucleoli, attached to the chromosomes. Meanwhile the chromosomes become diffuse and spread into the interior of the nucleus, finally condensing again on the periphery. The employment of various technical methods produced interesting variations in the aspect of oocyte chromatin and nucleoli.

The appearance of the Nebenkern in spermatocytes during the growth period was noted, but no indication of its presence discovered in oocytes until the fairly late growth period.

The chromosome forms were noted in spermatogonia, first spermatocytes and oocytes, and their numbers found to be 61-63, 31, and 31, respectively, but no clear dividing oogonia were found, on which similar observations could be made.

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INTRODUCTION

Facts concerning hermaphrodites are naturally of interest to-day, with sex phenomena holding a prominent place in biology. The theories of sex determination and differentiation which have been formulated to account for the conditions in dioecious animals have received no support from the group of hermaphrodites, because knowledge concerning the latter is almost wholly lacking. In some of the molluscs, where male and female germ cells are present in the same acinus and developing simultaneously, it seems that the opportunity for discovering the cause of the diverse differentiation of the two types of cells is quite favorable. We find, on taking up this major problem, that its solution rests upon the answers to several lesser questions. It is necessary to note the differences in the fully developed sexual products and trace the two lines back to a point where they meet—a procedure difficult to accomplish at times. Besides the common disadvantages of small cells and numerous chromosomes, other obstacles are present, due to the fact that there is no orderly arrangement of the members of the series with respect to time sequence and that the separation of the two lines becomes increasingly uncertain as they converge.

The literature on hermaphrodite molluscs is extensive, for the germ cells of these animals have been a source of interest to cytologists for the past fifty years. The first investigations, those of Duval, in 1878, and Blomfield, in 1881, resulted in a few observations on the grosser and more obvious features of the male cells. Additions to and corrections of the early literature accumulated. The appearance of the germ cells at various periods was described, the order of the stages suggested, and the origin of certain cell structures discussed

(Platner, '85, '86; Prenant, '87; Lee, '96, '97). The spindles and the Nebenkern, their respective origins and fates formed the subject of many papers (Prenant, '87; Lee, '96, '99; Murray, '98). Although, from the beginning, the contents of the nucleus were not slighted, they figure more prominently in the later writings. Platner ('86) had described the nucleoli of Helix and Arion oocytes; Zimmermann ('91), the mode of nuclear division in spermatogenesis; Obst ('99) contributed observations on the nucleoli of three mollusca. In 1910, a further addition to this section of the literature was made by Lams. In the work of Kleinert ('09), Soós ('10), and Demoll ('12, '13), and more recently, in those of Schitz ('25) and Crabb ('26) the chromosomes figure prominently. Baltzer ('13) attempted to determine on the basis of their chromosomes whether the offspring of a Tachea (Helix) hortensis individual mated to a Tachea austriaca were true hybrids, the product of self-fertilization or of parthenogenesis. From the time of Ancel's extensive investigation of the male and female germ cells of Helix (1902), the question of the differentiation of the cells has been of interest to cytologists. In no case has the necessary proof been presented to demonstrate that there is a relationship between sex production and chromosomes in hermaphrodite molluscs, or that the contrary is true.

MATERIAL AND METHODS

The material used for this study was Polygyra appressa, one of the land snails, and was collected from Burlington, New Jersey.

The shells of the snails were broken and the animals extracted. In some cases the snails had previously been etherized. The head of small animals and the anterior half of large individuals was cut off and as much of the alimentary canal pulled away as was possible without breaking up the digestive gland. The digestive gland and ovotestis were fixed whole in most cases. Some glands, which were to be fixed in Flemming's fluid or were intended for smears, were first cut into several pieces. Carnoy and Lebrun's fixative was used more extensively than any other fluid, because it fixed with greater precision the chromatin of adult glands, surrounded as they were by the digestive gland. Allen's B_{15} gave fair results. Flemming's strong fluid without acetic, used warm, was employed in fixing material for the study of the Nebenkern. Regaud's formol bichromate, Kopsch's and Champy's fluids were also tried, but without success. Carnoy and Lebrun's fluid was modified, being made up with one-quarter and one-half the usual amount of acetic acid, but the weaker fluids fixed the oocytes poorly and gave no advantages in other respects. Some smears were made of the ovotestis and were fixed in Flemming's fluid without acetic or in Carnoy-Lebrun's fluid. The remainder of the material was embedded in paraffin and cut into sections 6 μ to 8 μ in thickness. Most of the slides were stained in Heidenhain's iron haematoxylin; a few slides in Flemming's tricolor.

The majority of observations were made upon material fixed with Carnoy-Lebrun's fluid and stained with Heidenhain's haematoxylin, and when this work was completed some of the sections of Carnoy-Lebrun material were stained according to Feulgen's method (McClung, '29), using basic fuchsin and light green. New observations, based upon this later study, were added to the rest.

ACKNOWLEDGMENTS

The kindly assistance which Dr. C. E. McClung has given, in offering advice and criticism and in granting the use of the necessary laboratory facilities, is greatly appreciated by the author, who wishes to express her thanks to him at this time. She is also grateful to Dr. H. B. Baker for identifying the specimens used in this research.

OBSERVATIONS

A. Structure and composition of the hermaphroditic gland

1. General form of the gland and its location. The smallest individual which I have so far observed is one whose shell measured 5 mm. in width. The hermaphrodite gland of such an individual is a relatively small, simple structure with few acini. The ovotestis is embedded in the digestive gland near the columella and is just commencing to send out its branches among the lobes of that organ. In adult snails the sex gland compares quite favorably in bulk with the digestive gland, the two organs occupying most of the upper portion of the shell. The hermaphroditic gland of an adult consists of numerous acini which extend from the inside of the coil to its periphery. Polygyra appressa is quite similar to Helix in respect to the general structure of its reproductive gland.

2. *Composition of the young gland.* *a.* The ovotestis of Polygyra has as its outermost covering at all times a membrane, which at places can hardly be distinguished, owing to its extreme thinness. The cytoplasm of its cells is refractive in the fixed preparations and its plate-like nuclei stain deeply with haematoxylin (fig. 1, *m*). The layer of cells coming next to the membrane is usually one cell deep, but it may be more. The elements which constitute this layer are almost exclusively cells having deeply staining nuclei and scant cytoplasm (fig. 1, *w*). The dark aspect of the nuclei is produced by numerous, heavily stained blotches of chromatin crowded within their membranes; possibly to a lesser extent by the slightly tinged nuclear sap. In some of the lighter cells one or more irregular nucleoli may be seen. It is questionable whether these cells have individual cell boundaries or whether, as some of the investigators (Lee, '97; Buresch, '11; Gould, '17) have claimed for similar cells in Helix and Crepidula, they form a syncytium. At any rate, in Polygyra, when the cells are not crowded together very closely, individual cell boundaries can be discerned.

At intervals the regular line of the acinus wall cells is replaced by other kinds of cells. The most conspicuous of these are the young oocytes which appear singly and are easily recognized by their large size and characteristic nuclei and nucleoli (to be described later). The number of such cells which I counted in a gland of a 5-mm. individual was about eighteen or twenty. Here and there, the row of wall cells bulges into the acinus and the space between them and the membrane is occupied by a group of fairly large cells (seldom a single cell) which may be all of one type or of different types. The most abundant kind resembles a type found extensively in the lumen, distinguishable by dark, more or less regular chromatin masses spread over the surface of the nuclei (fig. 1, *og*). Less numerous are cells with large nuclei displaying the spireme or polarized loops and resembling similar cells of the sperm series, from which they differ only in location (fig. 1, *oc*). Rarely, one sees a single small mitotic spindle with closely packed chromosomes.

b. All cells lying within the lumen of the very young acinus have little cytoplasm; probably as a result of their recent rapid multiplication. Some of them are the spermatogonia. They are larger and lighter in aspect, not as closely packed together as are the wall cells. The blocks of chromatin are spread over the large, spherical nuclei and take up the haematoxylin to a greater or less extent in the various cells (fig. 2).

The type of cell just pictured is likewise found in the acinus wall. Moreover, there are cells like it occurring singly or in small groups in the older glands, more frequently in the periphery of the acini than in their interior. It has been one of the most difficult types to interpret with certainty, but its characteristics seem to limit it to the gonial generations or even earlier. Particularly favorable to this interpretation is its appearance in greatest numbers early in the ovotestis, its relatively undifferentiated aspect, and its general distribution.

In comparing this gland with that of another snail of like shell dimensions but whose gonad, judging by its general proportions and the number of types of cells it contains, is a little more advanced than the former, I have found that there are some spermatogonia in the older gland, but that they do not numerically predominate as in the younger gonad. Their place is taken by a number of smaller cells with resting nuclei or with polarized chromatin loops, both kinds being frequently arranged in radiating groups; whereas the cells which have been diagnosed as spermatogonia lie independent of one another. The radial arrangement comes later than the unorganized condition. This observation gives us an additional means of recognizing the spermatogonia.

The composition of the young sex gland has been described here at length for two reasons. A knowledge of its contents is essential in determining when, in the germ-cell history, the differentiation of the two kinds of sex products takes place. In the past, those who have described hermaphrodite molluscs have disagreed widely with each other in respect to the composition of the young germ gland and have based their theories of sex differentiation on these observations. Of secondary interest is the problem of determining the exact time sequence of certain stages in spermatogenesis—a subject which will likewise profit by a comparative study of glands of various ages.

3. Composition of older glands. Corresponding to the size increase and complexity of form of the ovotestis there occurs a progressive increase in the number of different stages which are present, until in the adult we find, in addition to the types of cells already described in the 5-mm. individuals, all later stages of developing sperm, and of the ova up to the prophase of the first oocyte. As in Helix, groups of developing sperm in about the same degree of differentiation are frequently clustered about a basal cell, or more rarely, single male cells are present. Single ova with their nurse cells are also there. A considerable number and variety of stages are encountered in the same acinus. One observes no orderly arrangement of the groups of cells undergoing spermatogenesis or oogenesis either with respect to each other or to the general topography of the gland, except that the developing ova always lie in the acinus wall or next to the membrane which covers the gland.

In young snails which have not reached their full growth, the germ cells lie closely packed together and a group composed of cells all approximately in the same phase of development is hemmed in by neighboring clusters of cells. In the acini of fully mature or old snails, where some of the sperm and eggs have already been discharged, there are empty spaces, and the fan-like sections of bunches of young male cells radiating from points in the acinus walls are often well separated from one another.

B. The development of the oocyte

1. Characteristics of the well-developed oocyte. Cells which can be identified unmistakably as oocytes by other means are always to be found in the acinus wall. The location of the sperm cells is successively the wall of the acinus, then the lumen, and not vice versa. If we regard location as the single criterion for determining the sex of the young germ cells, then those cells resembling spermatagonia and young spermatocytes found in the acinus wall must be considered female cells.

Taken in conjunction with location, however, the following characteristics serve to distinguish the ova from cells in the male series: 1) a relatively large amount of cytoplasm as compared with male cells having somewhat similar nuclei; 2) frequently, the distribution of the cytoplasm in such a way that the resultant cell is ovoid and its nucleus is similarly formed; 3) a prominent nucleolus in young oocytes and a double nucleolus in later stages; 4) the presence of nurse cells which are attached to the oocyte; 5) the fact that the female cell is single or one of a very small group of cells.

2. The oocyte during its development. I have roughly divided the development of the oocytes into a number of periods, based upon the most important observable changes in them. Since the size of the cell and nucleus is one of the important indications of the growth period and convenient to designate, I have used it almost exclusively to indicate the various phases of development. The dimensions are in every case those of a given cell, but may be taken as a rough approximation of the average of a group at that particular stage.

a. Oocyte 22 μ in length with nucleus 9 μ in diameter (fig. 3). The young oocytes, usually single, are to be found in individuals of all sizes from those 5 mm. in width to fully grown adults, but in greatest numbers in snails not quite fully grown. Such an oocyte is pictured in figure 3. It possesses the characteristic ovoid shape. As one sees the cytoplasm in the fixed preparations it seems to be as yet undifferentiated. There is a single large nucleolus, stained black with haematoxylin, and usually with chromatin strands attached to it. For its appearance when other methods of technique are used see table 2. The remaining chromatin lies in the outer portion of the nucleus and is contained in fairly regular masses with definite outlines, often suggestive of paired chromosomes.

b. Oocyte 25 μ in length with nucleus 18 μ long (fig. 4). Cells with these dimensions are found in individuals of all ages. No change has taken place in the cytoplasm except an increase in amount. The nucleolus has about doubled its bulk, and now measures 3.8 μ. Sometimes, at this period, small variations in staining capacity suggest that it is not homogeneous. The masses of chromatin which are still near the surface of the nucleus appear more decidedly chromosome-like, probably because in their distribution they show more readily as individuals. Their bipartite or quadripartite nature is plainly visible. The chromatin is less condensed than formerly, the chromosome outlines more irregular.

c. Oocyte 41 μ in length with nucleus 23 μ (fig. 5). The nucleolus measures 5 μ in diameter and shows plainly that it is losing some of its staining capacity—a change which continues straight through the growth period. During the transition period the nucleoli present various aspects (figs. 6 and 7). Perhaps the most important feature of this stage consists in the appearance on the old nucleolus of another smaller body which stains deeply with haematoxylin. This structure, after its first appearance, seems to be a permanent part of the nucleus while the oocyte is in the acinus wall. The second nucleolus is subspherical in shape and lies slightly flattened against the plasmosome, this being its usual location. Later on, it may be observed occasionally detached from the plasmosome.

The chromatin appears as tangled threads bearing grains or lumps (fig. 8). A change in the location of the chromatin has taken place, resulting in its distribution in the interior of the nucleus as well as on the surface of that body.

d. Oocyte 52 μ in length, but varying within greater limits than formerly; length of nucleus, 27 to 37 μ. The plasmosome, varying from cell to cell, takes up the stain to a lesser degree than previously (fig. 9).

The chromatin has become so dispersed throughout the nucleus that individual chromosome outlines can no longer be traced. This phase in the development of the nucleus is probably brief, for there are few cells which show it; more often, one sees masses of the more compact chromatin in the periphery.

Up to this time, there has been a very small amount of yolk formed, but now, along with a marked increase in the cell dimensions, there is a parallel increase of the yolk.

e. Oocyte 60 μ in length; length of nucleus, 42 μ; plasmosome, 15 μ; secondary nucleolus, 2.5 μ (fig. 13). There is still no uniformity in the condition of the plasmosome revealed by its staining capacity in various cells, but its great size is a constant feature. Modifications in the form of weakly staining globules or uncolored areas are so frequently present in the plasmosome at this time in contrast with the uniformity in earlier and later periods as to suggest that quite a range of conditions is the rule and that the variations in the fixed and stained material are not wholly due to variations in the technique.

At some time between the stage which was last described and the present one, the limit of the deconcentration of the chromatin is reached and the opposite reaction sets in which causes the chromatin to return to the peripheral region of the nucleus and to collect in larger masses. We can now see most of the chromatin in large fluffy masses just beneath the nuclear membrane, as a first step toward its condensation. The karyoplasm evidently does not return to precisely its former condition, for there remains distributed throughout the nucleus a coarse, unstained network, bearing spherules of a substance staining in various degrees. In material fixed in Flemming's fluid without acetic or in B_{15} these globules do not stain at all, but in Carnoy-Lebrun material they are colored by the haematoxylin frequently as deeply as the chromosomes themselves.

The yolk fills a considerable portion of the cell, but does not completely surround the nucleus. There always remains at least a small amount of cytoplasm bordering on the nucleus. There can be distinguished in the yolk a number of patches of faintly stained, finely striated substance. The form of these areas is ovoid or spindle-shaped. They are probably portions of the undifferentiated cytoplasm which have become surrounded by yolk. In some of the latest oocytes they are no longer found.

f. Oocyte 212 μ in length with nucleus 61 μ; plasmosome, 21 μ, secondary nucleolus, 3 μ in diameter (fig. 12). It will be evident from a comparison of the dimensions of the earliest oocytes and this, the latest stage, which I have observed in the hermaphrodite gland of Polygyra, that the cell body, the nucleus, and the plasmosome have increased enormously during the interval; the secondary nucleolus, from the time of its appearance, only slightly.

The plasmosome is a fairly homogeneous, faintly stained, ovoid body, eccentrically placed in the nucleus. It seldom comes in contact with the nuclear membrane, although it often lies very close to it. One side of the plasmosome is indented by the subspherical secondary nucleolus. A departure from this condition is present in cells which show two secondary nucleoli or those in which the secondary nucleolus is detached from the plasmosome. I have never seen more than one secondary nucleolus attached to the same plasmosome; when the nucleus contains two, one or both lie free in the nucleus. Irregular, lighter or darker areas and sometimes a tiny granule or two can be distinguished in the plasmosome, but no regularly occurring structures. The clear areas suggest the clear, spherical area and its chromatin masses which formerly filled a portion of the plasmosome (fig. 9) and of which they may be all that remains.

The chromosomes are at or near the surface of the nucleus, and there are present in oocytes fixed in Carnoy-Lebrun's fixative and stained in haematoxylin black granules with sometimes fine connecting strands scattered throughout the nucleus. The chromatin contained in the chromosomes has again become condensed, as it was in the very early oocytes, but as long as the oocytes remain in the acinus wall the chromatin is not as compact nor has it as sharply defined outlines as the late prophase chromosomes of the first spermatocytes (fig. 12, *c*). Moreover, the oocyte chromosomes exceed in size the spermatocyte chromosomes, which never attain in their whole history the size of the egg chromosomes of this period.

As the oocyte chromosomes condense, their internal structure is in many instances displayed with distinctness, particularly in the B_{15} material (figs. 10 and 11). The chromatids, as long slender threads, have been visible for a long time previously, but during this brief period, when they shorten and become more compact, the components of the chromosomes are most clearly demonstrated. Many of the chromosomes finally take the form of two loosely joined elements, and on account of this fact it is difficult to make an exact count of their number. One cannot be certain in many cases whether one is counting two parts of a single, bipartite chromosome distributed in two sections of the material or two separate chromosomes. In sixteen counts of the oocyte chromosomes in individual cells the numbers range from twenty-nine to thirty-six. One can be fairly sure that the highest counts exceed the actual number, because the bipartite condition will tend to increase the counts. The number of gonads fixed well enough to render a satisfactory preservation of the late oocytes is small. The best cells are those which were subjected to Carnoy-Lebrun fixative. The counts in two such cells are each thirty-one chromosomes.

3. Comparison of nuclei treated with various methods of technique. The description which has just been given of the changes taking place in the growing oocytes has been based on a study of material fixed with Carnoy-Lebrun's fluid and stained with Heidenhain's haematoxylin. That which was observed in Carnoy-Lebrun material, mordanted in Flemming's fluid and stained with Flemming's tricolor, confirmed these results. However, the study of oocytes which had been fixed with Flemming's strong fluid without acetic acid and stained with Feulgen's stain or Heidenhain's haematoxylin, and also oocytes fixed with Carnoy-Lebrun's and stained according to Feulgen's method gave rise to some new observations. In order to set forth the contrasts which the various fixing and staining methods reveal, I have constructed table 1.

C. Spermatogenesis

1. The spermatogonia. a. Some of the cells with heavy chromatin masses in the outer layers of their nuclei, which are described as abundant in the lumen of the very young gonad and present in fewer numbers in older glands, have been interpreted as spermatogonia (fig. 2). The cytoplasm of the spermatogonia is meager; the cell measures 20 μ in diameter and has a nucleus 13 μ across.

b. As is usually the case in spermatogonia of animals possessing small cells and many chromosomes, the chromosomes in most cells of this type when undergoing mitosis are too crowded to enable satisfactory counts to be made upon them. However, a careful search revealed a few good cells. The chromosome number in one cell was found to be sixty-one or sixty-two (fig. A); in another cell, sixty or sixty-one. The chromosomes have the form of V's or rods, with the exception of a single individual which is a medium-sized sphere. Some of the rods are clearly bipartite, some are bent.

TABLE 1

Comparison of nuclear conditions in Polygyra appressa oocytes as revealed by various methods of technique

PERIOD IN DEVELOPMENT	TECHNIQUE		NUCLEOLI	CHROMATIN
	Fixative	Stain		
Early growth	Carnoy-Lebrun	Heidenhain's haematoxylin	One black nucleolus grows and becomes lighter. Second nucleolus appears attached to first	Thick, dark blocks, often paired or quadripartite
		Feulgen's with light green	One green	Pale purple blocks
	Strong Flemming without acetic	Heidenhain's haematoxylin	Same as with Carnoy-Lebrun and Heidenhain's haematoxylin	Gray blocks
		Feulgen's with light green	Pale orange or tan nucleus	Green blocks
Late growth	Carnoy-Lebrun	Heidenhain's haematoxylin	One large, unstained or faintly tinged plasmosome attached to a smaller, black nucleolus	Gray diffuse chromatin; slender, black chromatin strands; and finally, well-condensed, black, prophase chromosomes
		Feulgen's with light green[1]	One large, pale green or uncolored nucleolus and a variable number of smaller, green nucleoli, attached to some chromosomes	Green chromosomes with purple cores or threads. (This, the latest stage in my material, may not be the latest observable in the ovotestis)
	Strong Flemming without acetic	Heidenhain's haematoxylin	Two black nucleoli, attached (comparable in size to the pair in the Carnoy-Lebrun material), and a variable number of black nucleoli, attached to some chromosomes until the chromosomes condense	Large, light gray, strand-like accumulations of chromatin finally condense to smaller, dark gray heaps with black, often tetrad-like centers
		Feulgen's with light green	One large, orange or brown nucleolus, and one pale orange or colorless, smaller nucleolus attached to the first one. Pale orange nucleoli attached to some chromosomes	Green

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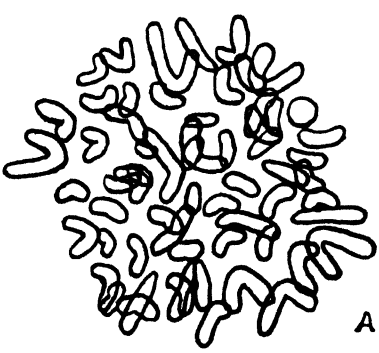
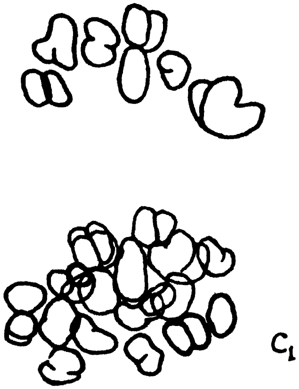
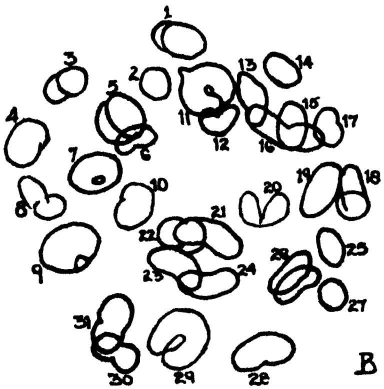
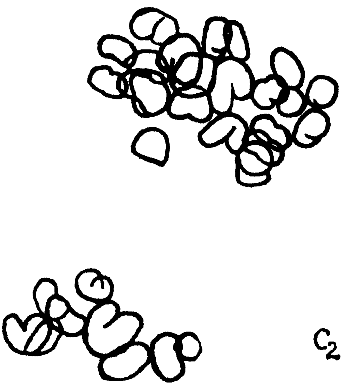
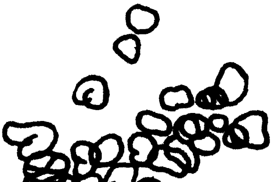
[1] Observations unsatisfactory because of insufficient material.











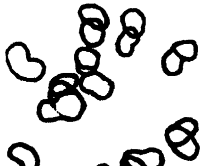


Fig. A Polar view of the spermatogonial chromosomes in metaphase.

Fig. B First spermatocyte chromosomes in prophase, from a smear preparation.

Fig. C 1 and 2. Group of first spermatocyte chromosomes in anaphase (side view). Cell cut in two sections.

Fig. D 1 and 2. Group of second spermatocyte chromosomes in two sections. Side view of anaphase.

2. The first spermatocytes. A comparison of the spermatogonia with the earliest members of the spermatocyte series shows that the spermatogonia exceed the latter cells in size and that they more nearly approach a spherical shape, whereas the spermatocytes are pear-shaped and lie in clusters with their apices approaching one another.

a. Figures 14 to 19, inclusive, show individual cells, each one of which belongs to a fairly well-isolated group of its kind projecting from the wall of an acinus which contains all of them. I find that, by taking into consideration the cell size, arrangement, and staining capacity of the chromatin of the cells of each of these groups, the types form a series, the further discussion of which will be taken up under the section devoted to discussion. A statement of the order of the stages and some brief explanatory remarks will be given at this time. The first member of the series (fig. 14) has a large nucleus, closely packed with a tangled network of chromatin, bearing heavy knots, and as a rule one or two spherical or irregular nucleoli. The amount of cytoplasm contained in the cell varies, the cell being pear-shaped if much is present. The following phase (fig. 15) is characterized by slightly larger cells containing masses of threads, the individual strands of which can at times be made out with some distinctness.

Two alterations appear in the nucleus before the next phase; consequently the chromatin is contained in loops lying parallel to one another with their ends directed toward one side of the nucleus. A variation, at times visible, shows the threads are polarized but not double, or double only a part of their length, thus displaying the true bouquet condition (figs. 16 and 17). The double chromatin threads are later seen freed from their orderly arrangement and scattered through the nucleus (fig. 18). The following stage shows a resting condition of the nucleus with the chromatin in the form of a delicate network, bearing granules and a deeply stained sphere, the nucleolus, with protruding knobs of chromatin (fig. 19). The resting cells are large in comparison with earlier members of the series.

b. When the resting state is over, the male cell has reached its maximum size with a length of about 21 μ and nuclear diameter of 13 μ to 16 μ. The chromatin again stains readily with haematoxylin. The chromatids of the individual chromosomes appear first as long slender threads twisted about each other, but the tetrad nature of the chromosomes is most conspicuous as the threads contract and become thicker (fig. 20). After nearly all of the chromatin is gathered into the prophase chromosomes, faintly tinged linin threads, on which are strung a few granules, join the chromosomes one with another. While these alterations in the chromosomes are taking place the nucleolus can for some time be discerned (fig. 20, *n*), but when the chromosomes have become well defined and homogeneous the nucleolus cannot be distinguished.

A count of the first spermatocyte chromosomes is most advantageously made during the late prophase, since the chromosomes are at this time well condensed and lie as far apart from one another in the nucleus as they can get. The same difficulty arises in counting the spermatocyte chromosomes as in making counts of the oocyte chromosomes, viz., uncertainty due to the incomplete fusion of the parts of single chromosomes. In thirty-one counts made of spermatocyte chromosomes the numbers range from twenty-seven to thirty-three. In two cells in which the counts were made under exceptionally favorable conditions I found thirty-one chromosomes (fig. B).

The predominant basic form of the chromosomes is that of a pair of rods. They differ from one another in the proximity of the two parts, the angle which these parts make with each other, and in the amount of bending of the rods. Out of the thirty-one members of the group of chromosomes, three or four are closed rings. The smallest chromosomes usually appear as a pair of closely joined spherules; the largest, as rings or loosely connected rods. Rarely can one see the true tetrad structure of the chromosomes in the latest prophase condition; one should look for it in earlier or later periods.

c. When the chromosomes have arranged themselves in the equatorial region, it becomes impossible to see and count all of them (figs. 21 and 22). The form of the majority of the metaphase chromosomes is that of two closely connected spherules, placed upon the spindle with their dividing plane parallel to the equatorial plate. Form differences between individual chromosomes are slight and there is nothing to indicate the presence of the single round chromosome of the spermatogonium, either in its earlier form or as a part of an irregular chromosome.

d. In division, the severance already indicated in the prophase is completed and the two daughter chromosomes go to the poles (figs. 23 and 24). Uusually, the division and the subsequent approach to the poles are not simultaneous in all of the chromosomes—a circumstance which results in a scattered anaphase group (fig. C). Most of the daughter chromosomes show the split between the two chromatids, and the individuals which result from the halving of a ring look like double V's. It seems that there is at least a partial fusion of the chromosomes in the telophase of the first spermatocyte and probably a resting nucleus is formed before the second spermatocyte division.

3. The second spermatocytes. The final determination of the second spermatocytes, as distinguished from firsts, must rest primarily on the form differences of the largest chromosomes, since size differences between the two types of cells are often inconspicuous, and sizes and shapes of the majority of the chromosomes in firsts and seconds uniform. Ring chromosomes are absent from the second spermatocyte spindles. The largest members of the complex are double V's, whose planes lie parallel to the equatorial plate, instead of at right angles to it as did the largest chromosomes of the first maturation spindle. Moreover, there seems to be less precision in the positions of the chromosomes in the second maturation spindle, resulting in a wider, more scattered figure with chromosomes at all angles to the plate (fig. D). The anaphase figures show single V's and smaller, bilobed chromosomes.

D. The Nebenkern

The youngest male cell in which I have been able to detect the Nebenkern contains a nucleus preparing the skein prior to its polarization. Before this time, the male germ cells contain scarcely any cytoplasm and no Nebenkern is visible. The Nebenkern, on its first appearance, has the form of a hemisphere and is closely applied to the nuclear membrane (fig. 25). In the polarized chromatin loop stage it keeps this position, but during the resting stage which follows, it migrates to the adjoining cytoplasm, where it becomes more complicated in form, sending out projecting globules. In the early prophase of the first spermatocyte, it is more prominent than at any other time and during this period it looks as if it consists of a mass of bubbles (fig. 26).

I have not seen the Nebenkern in the youngest oocytes, as they contain very little cytoplasm and the density of the stain obscures structures in them. In some large oocytes there is a darkening of the cytoplasm at one side of the nucleus.

DISCUSSION

A. The differentiation of the germ cells

1. In hermaphrodites, such as Polygyra, having male and female germ cells developing simultaneously in the same acinus, the transformation of the sex products into male and female cells must necessarily take place some time within the period when the gland is first formed and the germ cells display their fully developed characteristics. At the earlier period, we have present one (Woods, '25) or several (Ancel, '02) similar nucleated cells originating from the mesoderm. At the later time, the two types of cells can be told apart by a number of distinguishing marks. There is little or no disagreement among the authors as to what prevails at these two end periods even in diverse species, but the accounts vary in describing the interval between them.

For the cytologist, there are obviously two general possibilities respecting the differentiation of the male and female cells. Either some factor or factors, which can be determined by observing the gland during its development from the simple to the complex state, arise and are responsible for the differentiation; or, what is theoretically as plausible, the cause of the differentiation is not one which can be revealed by cytological methods. In the second case, the various distinguishing marks of male and female cells owe their incitement to this hidden cause, but they themselves are not the conditioning factor.

2. A scrutiny of the germ cells during this critical period has convinced investigators that various factors are the true cause of the determination. Nevertheless, it is generally admitted that the cause remains undiscovered or undemonstrated. This statement applies to Helix as well as to forms receiving less attention.

Although the earliest observations on the germ cells of hermophrodite molluscs were made about 1878, it was not until 1902 that the mode of differentiation of the male and female cells was discussed. In this year, Ancel, working with Helix pomatia, described in considerable detail the early period of development of the hermaphrodite gland. He distinguished three separate phases in the history of the gland. The first of these is that of the origin of the progerminative male cells from the indifferent sex cells. The second phase consists in the appearance of the nurse cells at the expense of the indifferent cells. The third phase is concerned with the development of female cells from the indifferent cells. To the presence of the nurse cells is attributed the rôle of orienting the indifferent cells in the female direction—cells which in the absence of the nurse cells would have become male.

Buresch ('11), describing Helix arbustorum, was the next investigator to consider the subject of the determination of the sexual elements in Helix. Like Ancel, he attributed to the nurse cells the important function of determining which cells will become male and which female germ cells. The proximity of nurse cells determines which indifferent germ cells fall into the lumen and become sperm and which stay in the wall and become eggs. However, Buresch did not agree with Ancel in finding sperm only in young snails and eggs in older individuals; but rather, spermatogonia, nurse cells, and egg cells, all developing continuously and simultaneously.

Demoll ('12, '13), dealing with the same species of snail as did Ancel, states that development is parallel in male and female cells until the bouquet stage is reached. At this time, the Nebenkern is said to be thrust out from the nucleus and a little later the egg begins to grow rapidly and the chromatin of both kinds of germ cells shows a specificity. The specificity of the Nebenkern is directly dependent on the condition of the accessory chromosomes, which, although they are visibly alike, may possess qualitative differences. The reasons for including the accessory chromosomes in the scheme are that in dioecious animals the accessories are connected with sex determination, and that, at the time when the Nebenkern is thrust out from the nucleus, the winding about of the chromosomes is most intimate. In discussions of Demoll's work, especially when his theory of sex differentiation of the germ cells is being compared with those of other authors, the emphasis is placed on the part which the Nebenkern plays in his plan. In order to give exact expression to Demoll's views he is quoted ('12–'13, p. 84):

> Bei den Zwittern ist ja die Geschlechtsbestimmung indirekt auf äussere Faktoren angewiesen, und man kann sich nun fragen: wird durch diese bewirkt, dass verschiedene Nebenkerne entstehen, oder wird immer derselbe gebildet, in diesem aber durch äussere Faktoren eine Anlage hintangehalten? Beobachtungen an Helix geben hier keine bestimmte Antwort.

3. *a*. I have outlined in brief the principal solutions which have up to this time been offered for the problem of the differentiation of the germ cells in hermaphrodite molluscs. It is perhaps worth noting that all of the accounts are unanimous in including outside factors, as either the main or accessory agents in differentiating the germ cells. In addition to Demoll's statement, two others may be cited. Ancel ('02, p. 512) wrote:

> . . . . la cellule sexuelle primitivement indifférente s'oriente dans le sens mâle ou dans le sens femelle suivant les conditions q'elle rencontre dans la glande génitale au moment de son apparition. La présence d'un matériel nutritif spécial élaboré par les éléments nourriciers la détermine dans le sens femelle, son absence dans le sens mâle.

Buresch ('11, p. 327) expressed his views in the following words:

> Auf Grund dieser Abhängigkeit zwischen Ei- und Nährzelle können wir sagen, dass die günstigen oder ungünstigen Ernährungsbedingungen der sich entwickelnden Keimzelle die Ursachen sind die sie zwingen, nach männlicher oder weiblicher Richtung sich zu entwickeln.

In a discussion of the present question, some attention should be devoted to another factor, viz., the Nebenkern, which has been regarded in the past as conditioning the sex of the germ cells of hermaphrodites. However, it seems that even in Demoll's work, where it is stated that "das Geschlecht der Keimzellen wird stets von dem Nebenkern bestimmt" ('12–'13, p. 82), we find no evidence for looking upon the Nebenkern as a mechanism, such as the sex chromosomes, automatically determining which cells will become male and which female germ cells. In plate 4, figures 1, 2, and 3, of Demoll's '12–'13 publication there are pictured oocytes and spermatocytes, the determination of which was made by the author prior to the appearance of the Nebenkern in those cells.

b. It is difficult to decide whether the presence or absence of the nurse cells in the vicinity of the indifferent germ cells determines which cells will become male and which female germ cells; or, on the other hand, whether the presence of the nurse cells is but a necessary condition for the full development of the germ cells, once the determination has been made through some other means. Demoll describes and pictures a cell which he says is a poorly developed egg, unprovided with nurse cells ('12–'13, p. 85, fig. B). If this cell is really a poorly developed female cell, the evidence which it provides is in favor of the theory that the nurse cells are not the initial or sole determining agent.

4. I can trace the cells in the male line of Polygyra back to the spermatogonia, and in the female line to the young oocytes and possibly to the oogonia (see the section on oogonia, p. 438). If the gonia-like cells in the wall are female cells, then the following statements hold. At the gonial stage of their development the two kinds of cells are indistinguishable from each other by means of aspect, their location in the lumen or in the acinus wall alone making possible their identification (compare fig. 1, *og* and *oc*, with figs. 2, 17, and 18). I have not found an oogonium with chromosomes spread out in such manner as to display the individual members of the group, so could not determine whether the oogonia possess the single spherical chromosome which the spermatogonia contain. It may therefore be said in regard to Polygyra appressa that there is present, as far as I can determine, in the male and female germ cells, when they are first recognizable as such, no visible structure to which may be attributed the function of causing sexual differentiation.

The possibility remains for proving that the incitement of the peculiar characters of egg and sperm is due either to, *a*) the position of the cell, in which case there should be also a demonstration of the nature of the influences which bear upon the cells by reason of their location; whether these influences be nutritive or something else; or, *b*) some other cause or causes not yet suspected.

c. We can only conjecture at present which of the two possibilities mentioned will prove to be reasonable. It seems to me a logical attitude, considering our present knowledge, to look upon the germ cells as sexually undifferentiated cells which, through the influence of their surroundings upon their internal structure, take one or the other course of development. The actual differentiation of the germ cells is a slow, progressive change, its course along either the male or female lines being often predictable by means of the cell position, long before it actually sets in. If we call to mind what we know concerning the differentiation of the germ cells in dioecious animals, we shall not be surprised in failing to discover at the commencement of development sharp morphological differences, such as dissimilarity in chromosome form or in chromosome numbers, in the hermaphrodite. The sperm (or ova) of an animal may be of two kinds, these types displaying no differences except in the number or form of their chromosomes, whereas the ova of the same species may be distinguished from the sperm, when both are mature, by a variety of characters and at the same time possess a chromosome complex which appears identical with that of half the sperm.

As regards the time of the development of the two sorts of germ cells in Polygyra, I can say that in the smallest individual which I have examined (width of shell, 5 mm.) both male and female cells were already present. They occur in snails of all ages, but ova are more prominent and probably relatively more abundant in older animals. The maturation divisions of the egg do not take place in the ovotestis. I have found no stage later than the prophase of the first maturation of the ova. In contrast to this condition, there are mature sperm in the gland long before a single egg has reached the latest point in its development which it will attain in the hermaphrodite gland.

B. Oogonia

1. There is a division of opinion regarding the presence of oogonia in pulmonates. Several workers in the field failed to find them. The oocytes have been described by Ancel as arising directly from indifferent cells without undergoing division; thus two stages corresponding to the progerminative male cell and the spermatogonium were omitted in the female line. Buresch said that the indifferent germ cells of the acinus wall become indifferent sex cells. If such an indifferent sex cell stays in the wall, it grows, becoming an oocyte without dividing.

2. On the other hand, a number of investigators have described oogonia in these snails. Demoll pictured the young female cells as dividing and the resulting oogonia passing

through phases similar to those of the males, but the sister cells developing at different rates. There are a small number of oogonia present in Crepidula, according to Gould. They appear in the normal course of development of the eggs in Enteroxenos östergreni (Bonnevie, '05). There is no doubt that this stage is present in the dioecious prosobranch mollusc, Paludina, which Popoff has described.

I began the study of Polygyra with the examination of adult snails and was at first able to recognize female germ cells in no earlier condition than that presented by the young oocytes, such as the one in figure 3. It is true that I saw, lying next to the acinus membrane, small groups of gonia, but had no means of telling that they belonged to the female line. Moreover, there is the additional fact that the gonia, and as I learned later, the youngest cytes, appear to exceed in size oocytes of the type pictured in figure 3. Consequently, it is possible to make up a complete series of cells, grading in respect to size and nuclear characters in such a way as one would expect to find if the conditions which Ancel described in Helix prevailed, i.e., the oocyte developing directly and without dividing from the indifferent cell.

Later, when I examined the ovotestes of very young snails, I saw groups of cells similar to the ones shown in figure 1 and having about the same dimensions as cells in like phases situated in the lumen. As the search for clear oogonial chromosome complexes was unsuccessful, no comparison of spermatogonial and oogonial chromosome groups can be made to aid in the identification. The phenomena in Polygyra which I have described give us some basis for supposing that the oocytès do not dev.elop immediately from the undifferentiated or even larger acinus-wall cells, but undergo divisions similar to those of the spermatogonia and pass through phases which the spermatocytes undergo before they proceed to their greatest growth.

C. Growth and differentiation of the oocyte

1. *a.* Although there is a much less extensive literature concerning the development of the egg than of the sperm, it is difficult to get a clear idea of this phenomenon from the accounts which have been given, unless the reader accepts a single description and ignores the others. The investigators have used different methods of technique, have described structures in the oocytes in different terms, and one cannot always tell if they have described the same periods of development. I am giving in the form of a table the significant features in the development of the oocytes of Helix pomatia as described by four observers (table 2).

b. The changes in the nucleus of the growing oocyte of Polygyra appressa have been related in detail, so it will suffice here to sum them up briefly. The transformations in the chromatin in general are simple. Starting with the chromatin concentrated in dense masses in the periphery, there is a deconcentration and distribution of this material throughout the nucleus. Afterward, the opposite tendency is manifested and continues until the oocyte leaves the ovotestis. As a result of the latter reaction, there are formed in the nucleus chromosomes, resembling in general form, but exceeding in size, the prophase chromosomes of the first spermatocytes. A nucleolus is present in the early oocytes and can be distinguished from the chromatin by its characteristic form and its homogeneity even in the Carnoy-Lebrun material, stained with Heidenhain's haematoxylin, in which it stains similarly to the chromatin. Later, a second, smaller nucleolus is seen applied to the first one, and the original nucleolus loses its affinity for haematoxylin in a variable manner. A variable number of other nucleoli may be present during the latter half of the growth period. Their size is about the same as that of the secondary nucleolus, but they differ from it in staining capacity. They always seem to be attached to a strand of chromatin until the chromosomes condense. The plasmosome increases enormously from the time of its appearance; the secondary nucleolus, comparatively little.

TABLE 2

Nuclear conditions in the growing oocytes of Helix pomatia

Investigator	PLATNER	OBST		ANCEL		DEMOLL
Methods	Flemming's chrom-osmic-acetic; some safranin, some haematoxylin	Sublimate; methyl green and borax carmine		Bouin's and Flemming's fluids; iron haematoxylin, Flemming's tricolor		**Four kinds of fixatives; Heidenhain's iron haematoxylin**
Observations	Nucleoli	Chromatin	Nucleoli	Chromatin	Nucleoli	Chromatin
	One Keimfleck (plasmosome?) round, with projections. Becomes homogeneous. It grows and vacuoles arise from it. Separation into dark and light regions	In lumps, red. Fine and distributed in nucleus. Clumped. Network of fine granules and few larger pieces	One (seldom two) regular outline, blue. More appear, and fuse to two large vacuolated nuclei (then one?) Red and blue structure appears attached to nucleolus. Nucleolus becomes pale, with many vacuoles. Small structure becomes red	Little round spots, 'nucléoles nucléiniens,' with radiating threads on periphery of nucleus. Network of large mesh. Breaks into homogeneous patches and gives off →	Several parachromatic nucleoli (stain like chromatin) with haematoxylin, but differently with tricolor. Fuse into one Parachromatic particles, which form accessory nucleoli. Accessory nucleoli fuse with principal nucleolus. It becomes cytoplasmic	Chromatin threads loosen after bouquet stage, individual chromosomes are visible. Concentration. Ring-shaped chromosomes with threads going out. Streams out of rings and is in broad strands composed of two fine twisted threads, staining feebly with haematoxylin

2. It is customary to look upon the differences in the staining capacity of cellular structures during a period of their history as indicative of alterations in their chemical composition. I am not prepared to say what relationship these changes in the nuclear contents of Polygyra appressa oocytes bear to somewhat similar series in other species of animals, but the comparative study, using various methods of technique, has led me to draw several conclusions bearing upon the subject. Comparative cytological studies of such structures as the nucleoli have little value unless the objects which they compare have been treated by similar methods of technique. Since the stipulated basis for such a study is at present lacking as regards the nucleoli of molluscs (or for any other group, to my knowledge), generalizations as to structure and function of these parts rest upon an insecure foundation.

There is one difference in the oocytes which I have studied and those that other workers have described, and which it may be well to mention. Most of the previous investigators, describing the growth period of the ova, have pictured the chromatin during the greater part of this time as not being organized in the form of chromosomes and fail to show the internal structure of the chromosomes at any phase. On the contrary, I find evidence in Polygyra (especially when Carnoy-Lebrun's fluid and haematoxylin are used to prepare the material for study) that the chromatin is organized into individual chromosomes during almost the entire growth period, the exception being the period of the greatest diffusion of the chromatin. The chromatids can be discerned for a considerable time.

D. The order of the early spermatocyte stages

As I have already stated in relating the changes which the male cells undergo previous to the first maturation division, the order of the stages which the cells pass through is a difficult thing to determine. Anyone who examines the hermaphroditic gland of Polygyra can understand why there are almost as many different accounts of this period in Helix as there are separate expositions of the subject, and not until we reach Demoll's description of spermatogenesis in Helix do we get a satisfactory account of the order. In nearly all cases the authors omitted to state the means by which they determined the order.

E. The polarized pachytene stage

There is disagreement as to the internal structure of the pachytene loops of the first spermatocytes. Demoll ('12) pictures them at this stage, in his figures 11 and 12 of plate 5, and describes the paired chromatin threads as being wound about each other spirally, so that the two strands of a single pair appear to cross many times in the full extent of the loop. I do not find such a condition in Polygyra appressa. The individual threads seem seldom to cross, and the chromomeres of the paired threads can clearly be seen as dark spots along the threads (fig. 17). Popoff ('07) and Soós ('10) found in Paludina vivipara and Helix arbustorum conditions similar to what I have seen in Polygyra.

F. Pre- or postreduction

It should be evident from the description which I have given of the first and second maturation divisions of the sperm that there are no grounds for forming an opinion as to the pre- or postreduction of the chromosomes (Carothers, '26). Similarly, none of the work which has been done upon hermaphroditic molluscs offers a satisfactory basis for making such a decision for the chromosome complexes of the species involved. Nevertheless, nearly all of the authors, describing the maturation divisions, favor one or the other as being the reduction division.

SUMMARY

1. Both male and female germ cells are present in the ovotestes of Polygyra appressa of all ages and sizes (from those 5 mm. in width on), but the sperm mature before the ova.

2. The female germ cells remain in the wall of the acinus during the entire period of their stay in the hermaphroditic

gland, while the male cells undergo their development in the lumen.

3. Until the beginning of the greatest growth period of the first cytes, the male and female cells cannot be distinguished from each other by their aspect, their position in the acinus wall or in the lumen being the only criterion of their identity.

4. The chromatin of the oocytes during its growth period slowly changes in form from the heavy chromatin blocks, lying in the periphery of the nucleus, to dispersed granules, scattered throughout the nucleus, and back again to large, fairly well-condensed, prophase chromosomes, located in the periphery.

5. The chromatin of the oocytes is organized into chromosomes throughout a large part of the growth period, and during part of this time the chromatids are plainly visible.

6. A single nucleolus is present at first in the early oocyte, and greatly enlarges and becomes plasmatic. A second nucleolus grows out from one side of the first nucleolus, but increases only slightly in size.

7. The number of the prophase chromosomes in the oocyte is thirty-one. There are sixty to sixty-two chromosomes in the spermatogonium, and thirty-one in the first spermatocyte.

8. The Nebenkern appears against the nuclear membrane of the young first spermatocyte, prior to the polarization of the chromatin threads. Its form, at first subspherical, becomes more complicated and the Nebenkern moves out into the cytoplasm.

BIBLIOGRAPHY

(* Not seen in the original.)

Ancel, P. 1902 Histogénèse et structure de la glande hermaphrodite d'Helix pomatia. Arch. Biol., T. 19, pp. 389–652.

——— 1902 Sur le determinisme cyto-sexuel des gametes. Glandes genitales d'Helix pomatia sans ovocyte. Arch. Zool. Exp. (3), T. 10, pp. lviii–lxiv.

——— 1903 Sur le determinisme cyto-sexuel des gametes. Arch. Zool. Exp. (4), T. 1, pp. cv–cxv.

Auerbach, L. 1896 Untersuchungen über die Spermatogenese von Paludina vivipara. Jenaische Zeit. f. Naturw., Bd. 30, S. 405–555.

*Babor, J. 1894 Über den Cyclus der Geschlechtsentwicklung der Stylomatophoren. Verh. d. Zool. Ges., 4. Vers.

Baltzer, F. 1913 Über die Chromosomen der Tachea (Helix) hortensis, Tachea austriaca und der sogenannten einseitigen Bastarde T. hortensis × T. austriaca. Arch. Zellf., Bd. 11, S. 151–168.

Blomfield, J. E. 1881 The development of the spermatozoa. Part II. Quart. Jour. Mic. Sci., vol. 21, N.S., pp. 415–431.

Bonnevie, K. 1905 Das Verhalten des Chromatins in den Keimzellen von Enteroxenos östergreni. Anat. Anz., Bd. 26, S. 374–387, 497–517.

Boveri, T. 1890 Zellenstudien, Bd. 3, S. 1–18. Jena.

Buresch, I. 1911 Untersuchungen über die Zwitterdrüse der Pulmonaten. Arch. Zellf., Bd. 7, S. 314–343.

Carothers, E. E. 1926 The maturation divisions in relation to the segregation of homologous chromosomes. Quart. Rev. Biol., vol. 1, pp. 419–435.

***Colgan, N.** 1918 On the reproduction of the common garden snail, Helix aspersa. Irish Nat., Dublin, vol. 27, pp. 165–170.

Conklin, E. G. 1902 Karyokinesis and cytokinesis in the maturation, fertilization and cleavage of Crepidula and other gasteropods. Phila. Jour. Acad. Nat. Sci., ser. II, vol. 12, pp. 1–121.

Crabb, E. D. 1927 The fertilization process in the snail, Lymnaea stagnalis appressa Say. Biol. Bull., vol. 53, pp. 67–108.

Demoll, Von R. 1911 Zur Spermatogenese von Helix pomatia. Zool. Anz., Bd. 37, S. 88–90.

———— 1912 Die Spermatogenese von Helix pomatia L. Zool. Jahrb. Suppl., Bd. 15, S. 107–140.

———— 1912–1913 Über Geschlechtsbestimmung im allegemeinen und über die Bestimmung der primären Sexualcharaktere im besonderen. Zool. Jahrb., Bd. 33, S. 40–94.

***Duval, M.** 1878–1879 Recherches sur la spermatogénèse des gastéropodes pulmonés. Rev. d. Sc. Nat., T. 7 et 8.

———— 1879 Recherches sur la spermatogénèse étudiée chez quelques gastéropodes pulmonés. Jour. Micrographie, T. 3.

Garnault, P. 1889 Sur les phénomènes de la fécondation chez l'Helix aspersa et l'Arion empiricorum. Zool. Anz., Bd. 12, S. 10–15, 33–38.

***Godlewski, E.** 1897 Über mehrfache bipolare Mitose bei der Spermatogenese von Helix pomatia L. Bull. Acad. Sc. Cracovie, S. 68–81.

Gould, H. N. 1917 Studies on sex in the hermaphrodite mollusc, Crepidula plana. Jour. Exp. Zoöl., vol. 23, pp. 1–52.

Griffin, B. B. 1899 Studies on the maturation, fertilization and cleavage of Thalassema and Zirphaea. Jour. Morph., vol. 15, pp. 583–634.

Hertwig, O. 1878 Beiträge zur Kenntniss der Bildung, Befruchtung u. Theilung d. thier. Eies. Morph. Jahrb, Bd. 4, S. 197–210.

***von Ihering** 1875 Über die Entwicklungsgeschichte von Helix. Jenaische Zeit. f. Naturw., Bd. 11.

Jordan, H. E. 1910 A cytological study of the egg of Cumingia. Arch. f. Zellforsch., Bd. 4, S. 243–253.

***Kjerschow-Agersborg, H. P.** 1921 Contribution to the knowledge of the nudibranchiate mollusk, Melibe conina (Gould). Amer. Nat., vol. 55, pp. 222–253.

KLEINERT, M. 1909 Die Spermatogenese von Helix (Tachea) nemoralis und hortensis. Jenaische Zeit. f. Naturw., Bd. 45, S. 445–498.

*KOSTENECKI, K. 1911 Ueber parthenogenetische Entwicklung der Eier von Mactra mit vorausgegangener oder unterbliebener Ausstossung des Richtungskörpers. Arch. f. micr. Anat., Bonn., Abt. 2, S. 1–62.

*KRAHELSKA, M. 1912 Reduktionserscheinungen in der Eiweissdrüse der Schnecken. Kraków Bull. Intern. Acad., S. 606–621.

*KUŠAKEVIĆ, S. E. 1914 Les chondriosomes de la spermogénèse de Cerithium vulgatum. Kiev Prot. Obšč. jest., 1914 (1915), pp. 43–44.

*LAMS, H. 1907 La structure de l'ovocyte d'Arion empiricorum pendant sa période d'accroissement. C. R. Ass. Anat., T. 9.

——— 1909 Les globules polaires de l'oeuf d'Arion empiricorum (Fer). Arch. de Zool. Exp. et Gén., T. 1, p. i.

——— 1910 Recherches sur l'oeuf d'Arion empiricorum (Fer). Mém. l'Acad. roy. de Belgique, coll. in (4) T. 2, pp. 1–144.

LEE, A. B. 1895 Sur le Nebenkern et sur la formation du fuseau dans les spermatocytes des Helix. La Cellule, T. 11, pp. 223–260.

——— 1896 Les cinèses spermatogénétiques chez l'Helix pomatia. La Cellule, T. 13, pp. 197–278.

——— 1899 Les sphères attractives et le Nebenkern des pulmonés. La Cellule, T. 16, pp. 47–60.

——— 1910 La réduction numerique et la conjugaison des chromosomes chez l'escargot. La Cellule, T. 27, pp. 55–74.

*LEONARD, R. E. 1918 Early development of Haminea. Publ. Puget Sound Biol. Sta., vol. 2, pp. 45–63.

LILLIE, R. R. 1901 The organization of the egg of Unio, based on a study of its maturation, fertilization and cleavage. Jour. Morph., vol. 17, pp. 227–292.

LINVILLE, H. R. 1900 Maturation and fertilization in pulmonate gasteropods. Bul. Mus. Comp. Zool. Harvard, vol. 35, pp. 211–248.

LUDFORD, R. J. 1922 The morphology and physiology of the nucleolus. Jour. Royal Micr. Soc., pp. 114–150.

*MACKAY, M. E. 1927 a Early cytological development in Cumingia. Proc. and Trans. Nova Scotian Inst. Sci., vol. 18(1), pp. 46–60.

*——— 1927 b A study of early development in Cumingia, with special reference to cytology. Ibid., vol. 17(1), pp. 46–60.

McCLUNG, C. E. 1929 Handbook of microscopical technique. P. B. Hoeber, New York.

MEVES, F. 1902 Ueber oligopyrene und apyrene Spermien und über ihre Entstehung, nach Beobachtungen an Paludina und Pygaera. Archiv f. mik. Anat. u. Entwick., Bd. 61, S. 1–84.

MONTGOMERY, T. H. 1899 Comparative cytological studies with special reference to the morphology of the nucleolus. Jour. Morph., vol. 15, pp. 265–583.

MURRAY, J. A. 1898 Contributions to the knowledge of the Nebenkern in the spermatogenesis of Pulmonata, Helix and Arion. Zool. Jahrb., Bd. 11, S. 427–440.

OBST, T. 1899 Untersuchung über das Verhalten der Nucleolen bei der Eibildung einiger Mullusken und Arachnoïden. Zeit. f. wiss. Zool., Bd. 66, S. 161–213.

*ORTON, J. H. 1909 On the occurrence of protandric hermaphroditism in the mollusc Crepidula fornicata. Proc. Roy. Soc. London, vol. 81 B, pp. 468–484.

PELSENEER, P. 1894 Hermaphroditism in Mollusca. Quart Jour. Micr. Sci., vol. 37, pp. 19–46.

*———— 1910 L'hermaphroditism chez les Lamellibranches. Verh. intern. Zool. Kongr. Jena, T. 8.

PLATNER, G. 1885 Ueber die Spermatogenese bei den Pulmonaten. Arch. micr. Anat., Bd. 25, S. 564–581.

———— 1886 a Ueber die Entstehung des Nebenkerns und seine Beziehung zur Korntheilung. Ibid., Bd. 26, S. 343–369.

———— 1886 b Zur Bildung der Geschlechtsprodukte bei den Pulmonaten. Ibid., Bd. 26, S. 599–621.

———— 1889 Beiträge zur Kenntniss der Zelle und ihrer Theilungserscheinungen. Ibid., Bd. 33, S. 125–152.

POPOFF, M. 1907 Eibildung bei Paludina vivipara und Chromidien bei Paludina und Helix. Arch. mikr. Anat., Bd. 70, S. 43–129.

PRENANT, A. 1887 Observations cytologiques sur les éléments séminaux des gastéropodes pulmonés. La Cellule, T. 4, pp. 135–177.

*PROWAZEK, S. 1900 Spermatologische Studien. 1. Spermatogenese der Weinbergschnecke. Arb. Zool. Inst. Wien, Bd. 13, S. 197.

SCHAPIRO, J. 1902 Uber Ursache und Zweck des Hermaphroditismus. Biol. Centr., Bd. 22, S. 97–108, 135–151.

SCHITZ, V. 1916 Spermatogenesis in Columbella. Arch. Zool. Exper., T. 56, Notes et Revue, no. 2, pp. 32–47.

———— 1920 Spermatogenesis in gastropods. Ibid., T. 58, pp. 489–520

SMALLWOOD, W. M. 1904 Maturation, fertilization and early cleavage of Haminea solitaria. Bull. Comp. Mus. Harvard Coll., vol. 45, pp. 261–318.

———— 1905 Some observations on the chromosome vesicles in the maturation of Nudibranchs. Morph. Jahr., Bd. 33, S. 87–105.

SOÓS, L. 1910 Spermiogenesis of Helix arbustorum. Ann. Mus. Nat. Hungarici, vol. 8, pp. 304–343.

WASHBURN, F. L. 1894 Ookinesis in Limax maximus. Amer. Nat., vol. 28, pp. 528–531.

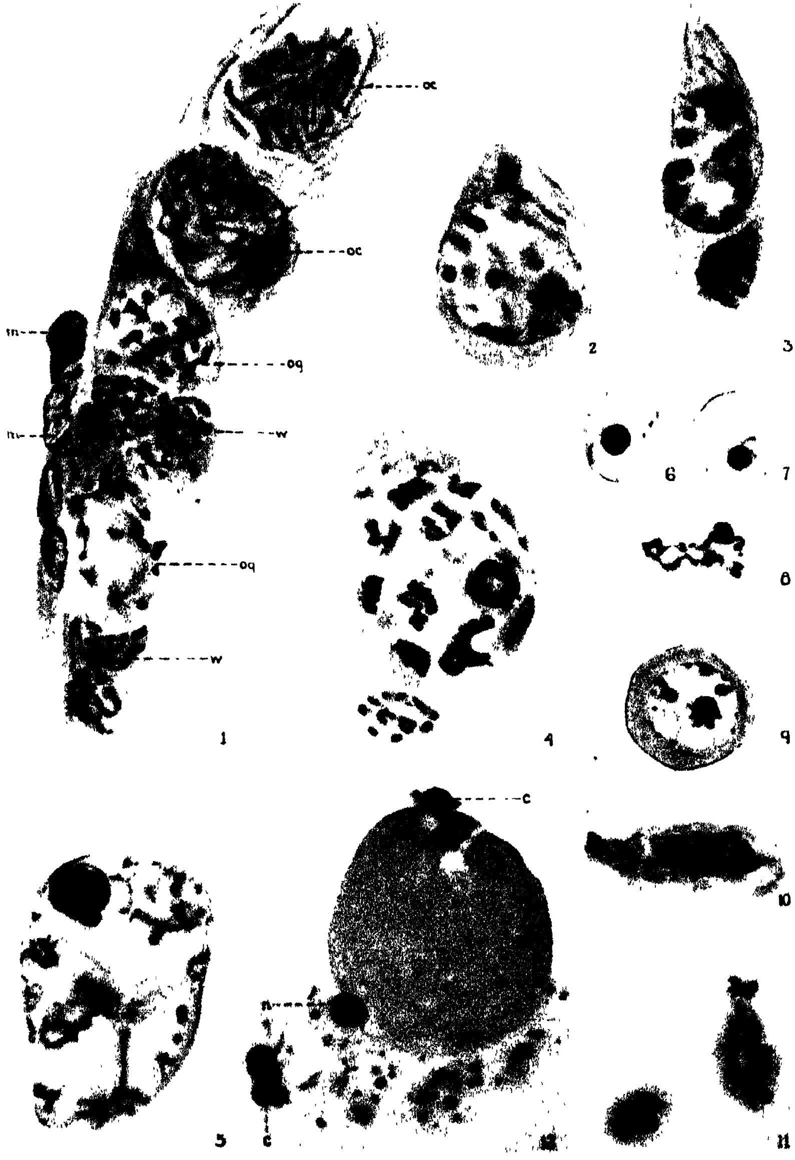
WILSON, E. B. 1925 The cell in development and heredity. The Macmillan Co., New York.

WOODS, F. H. 1925 History of the germ cells in Sphaerium striatinum. Anat. Rec., vol. 31, p. 305.

*ZARNIK, B. 1910–1911 Über den Chromosomencyclus bei Pteropoden. Verh. deutsch. Zool. Ges. Vers., Bd. 20–21, Leipzig, S. 205–215.

———— 1913–1914 Über die Diminution des Chromatins im Ei von Creseis (Pteropoda). Commun. 9eme Intern. Zool. Monaco, S. 272–277.

*ZIMMERMAN 1891 Über den Kerntheilungsmodus bei der Spermatogenese von Helix pomatia. Verh. anat. Gesel., Bd. 5, S. 187.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EXPLANATION OF PLATES

All figures have been reproduced from drawings made with the following equipment: Spencer microscope, 1.8 achromatic objective, 15× orthoscopic ocular; tube length, 165 mm. Reduction in reproducing the figures from the drawings: one-half. Magnification: × 1900 (i.e., one-half 3800, actual magnification of the microscope). Preparation of the material which these figures represent: unless otherwise stated, Carnoy-Lebrun's fluid, serial sections in paraffin, cut at 6 to 8 μ, stained with Heidenhain's iron haematoxylin.

PLATE 1

EXPLANATION OF FIGURES

1 Portion of the acinus wall of a snail, 5 mm. in width, showing: *m*, nuclei of the membrane surrounding the ovotestis; *oc*, oocytes; *og*, oogonia; *w*, wall cells.

2 Cell from the lumen of a 5-mm. individual.

3 An oocyte 25 μ in length, with nucleus 12 μ, accompanied by nurse cell.

4 An oocyte 25 μ in length, with nucleus 18 μ, with nurse cell.

5 Nucleus of oocyte, having length of 40 μ and nucleus 24 μ. Secondary nucleolus just appearing.

6 Nucleolus of 42 μ oocyte, having nucleus 26 μ.

7 Nucleolus of 57 μ oocyte with nucleus 24 μ.

8 Portion of chromatin from an oocyte nucleus of 25 μ.

9 Nucleolus of 52 μ oocyte, having nucleus 27 μ.

10 and 11 Chromosomes from an oocyte during late growth period. Nuclear length, 52 μ; cell length, 113 μ. Fixed with Allen's B_{15}.

12 Small portion of the nucleus of an oocyte in prophase, first maturation. Cell length, 212 μ; nuclear length, 61 μ. Secondary nucleolus, *n*, and one chromosome attached to plasmosome; another free chromosome, *c*.

PLATE 2

EXPLANATION OF FIGURES

13 Nucleus of oocyte 60 μ in length, having nuclear length of 42 μ.

14 to 19 Series of spermatocytes from the same acinus, fixed with Allen's B_{15}.

14 Resting stage.

15 Leptotene stage.

16 Bouquet stage.

17 Pachytene stage.

18 Diplotene stage.

19 Diffuse or resting stage.

20 Some chromosomes of a first spermatocyte nucleus, prior to late prophase stage. The nucleolus, *n*. Fixed in Allen's B_{15}.

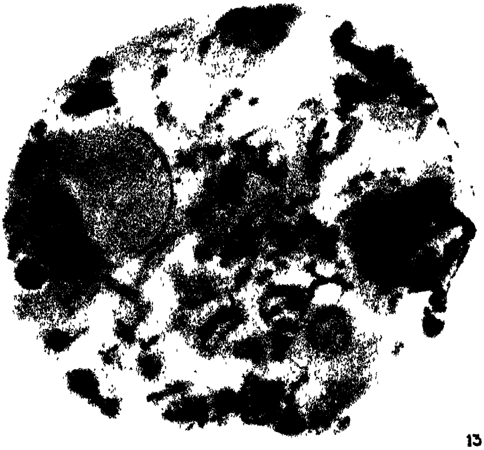
21 Side view of an equatorial plate of a first spermatocyte. Smear preparation, fixed in Flemming's fluid without acetic, stained with Flemming's tricolor.

22 Polar view of an equatorial plate of a first spermatocyte. All of the chromosomes are not visible.

23 and 24 First spermatocyte chromosomes just entering the anaphase.

25 Young spermatocyte in pachytene stage, showing the Nebenkern, *Nk*, against the nucleus. Fixative, Flemming's fluid without acetic.

26 A first spermatocyte in prophase with Nebenkern near the nucleus. Fixative, as for 25.



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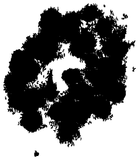

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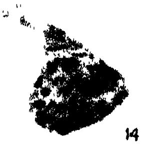

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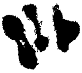

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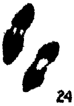

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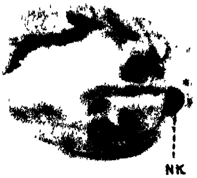

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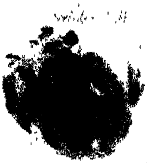

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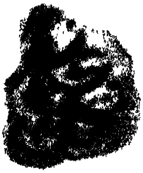

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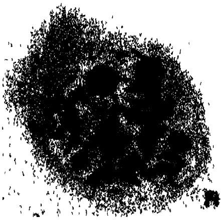

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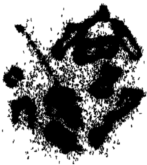

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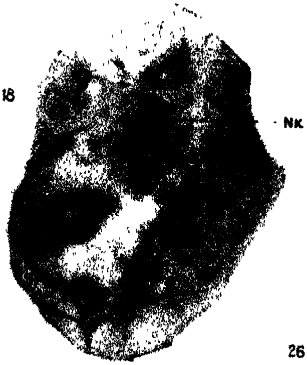

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CYTOPLASMIC PHENOMENA IN THE SPERMATOGENESIS OF GERRIS

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ONE TEXT FIGURE AND SIX PLATES (NINETY-THREE FIGURES)

AUTHOR'S ABSTRACT

The history of the cytoplasmic components in the spermatogenesis of Gerris is, in general, like that which has been described in the Pentatomidae. The observations of fixed material have been checked by extensive studies of freshly teased preparations. During the spermatocyte growth period the chondriosomes undergo considerable increase in mass. During the maturation divisions the chondriosomes are remarkably constant in orientation with respect to the centrioles. The nebenkern arises by fusion of chondriosomes differentiated into chromophilic and chromophobic portions. The Golgi bodies of the earlier spermatocytes are vesicular bodies, the peripheries of which are osmiophilic. These are not visible in fresh preparations, but the masses resulting from their fusion in the late prophase of the first division are visible in the unfixed cells. The non-osmiophilic material inside these masses stains with neutral red in fresh preparations Only the osmiophilic part of the Golgi masses is involved in the fragmentation to form dictyosomes. There is very suggestive evidence that the process of acrosome synthesis largely takes place inside the sac-like acroblast (Golgi apparatus). In the spermatid, material which stains, in fresh preparations, like the acroblast is never seen, except inside or attached to the acroblast, where it appears in the form of small spheres of 'pro acrosomic' material, which fuse to form the acrosome.

INTRODUCTION

An examination of Flemming-hematoxylin preparations of the testes of Gerris marginatus in the summer of 1926 indicated that the spermatogenesis of this form, although in general like that already worked out in detail for other Hemiptera, yet showed some unusual features that offered encouragement for future investigation. The most striking of these unusual features were the facts that the spermatozoa were of relatively enormous size and that the acrosome constituted a far greater proportion of the mature spermatozoon than is usually the case. Accordingly, an investigation of the history of the cytoplasmic constituents, especially of the mitochondria and Golgi apparatus, was undertaken by means of the special technical methods customarily used for their demonstration, including observations of freshly teased organs stained with vital dyes, in the hope that some minor problems in the history of these elements might be better solved in a form with such large spermatozoa.

I wish to take this opportunity to acknowledge my great indebtedness to Dr. R. H. Bowen for invaluable advice and criticism in the course of this work and to Dr. E. B. Wilson for many helpful suggestions.

MATERIAL AND METHODS

The material for this study was drawn entirely from members of the genus Gerris (Hemiptera-Heteroptera). I have relied mainly upon preparations of the testes of two species, Gerris remigis Say and Gerris marginatus Say, but I have also made casual comparison with three other forms, Gerris rufoscutellus Latreille, Gerris buenoi Kirkaldy, and Gerris canaliculatus Say. The identification of these species is due to the kindness of Mr. H. G. Barber. Gerris remigis was collected at Auburn, Maine; Plaistow, New Hampshire, and Bourne, Massachusetts. The other species were collected in the vicinity of Woods Hole, Massachusetts.

Although Flemming-hematoxylin preparations were useful for the general picture, I have relied mainly on special methods of demonstrating the cytoplasmic components for the study of details.

The Weigl method was used for the demonstration of the Golgi apparatus. The best preparations were obtained by fixation for twenty minutes followed by osmication for from five to seven days. Slides that were overblackened were bleached by turpentine or hydrogen peroxide. I have also employed a special method of my own that involves the use of a 0.5 per cent aqueous solution of the commercial antiseptic preparation, 'Zonite.' This is, I suppose, practically the same as Ludford's use of nascent chlorine for a similar purpose. With this method the bleaching can be controlled as carefully as the differentiation of an Fe-hematoxylin preparation. Such slides, however, are never so clear-cut as those in which bleaching was unnecessary. I have also tried the Kolatchev method for the demonstration of the Golgi apparatus, but with much less success.

For demonstrating the chondriosomes the best results were obtained by the methods of fixation and mordanting of Benda and Kull, followed by staining with either acid fuchsin-thionin-aurantia or alizarin-crystal violet.

With all the fixatives used there is considerable shrinkage of the cells. The poles of the dividing cells are especially poorly fixed, the general effect being that of a diffuse area, outside the centrioles, that appears to have burst out of the cell membrane. Examination of freshly teased preparations shows that the spaces between cells are occupied by masses of small spheres of apparently homogeneous substance. When the cells are freed from the cyst, the spheres appear in thin strands, which are attached in two groups at the poles of the cells. This is represented somewhat diagrammatically in figure 83. The strings are actually several times the diameter of the cell in length and are frequently much twisted and intertwined. That they are fastened at the two poles is most evident when the cell is being swept along in a current. These processes fix very poorly—a fact which explains the appearance of the poles of the cells in fixed preparations. The drawings from the slides have all been made as if the cell wall were continuous around the more compact region at the poles of the cells, and the diffuse, poorly fixed area is usually omitted.

In addition to the technical methods mentioned above, I have given considerable attention to the study of fresh preparations. These were made by teasing out the testes in a drop of Ringer's fluid on a slide. This was usually done under a binocular dissecting microscope. Such material, with a cover-slip over it, is suitable for study for twenty or thirty minutes. I have tried the hanging-drop method, but I consider it less desirable, since a 2-mm. lens cannot be used with it. These teased preparations show a great deal of detail, especially if one is familiar with the more obvious picture presented by fixed and stained material. Practically all the more important aspects of this study have been checked by this method, as will be explained later.

In connection with studies of fresh preparations it is advantageous to use vital dyes, especially neutral red and Janus green. Neutral red (rouge neutre Microcolor) in dilute solutions in Ringer's fluid can be applied either by immersing the entire testis in the solution for fifteen to thirty minutes or by teasing the organ apart in a drop of the same solution on a slide. I have not used any definite concentration, merely dissolving a small amount of the dye to give a solution of a light orange-red color. Janus green may be similarly applied in solutions that are light bluish-green in color. This latter dye is not likely to be too dilute, as it is said to stain in a strength of one part in 500,000. I have frequently mixed the two dye solutions and immersed the entire testis in the mixture—a procedure that seems to give practically the same result as when each dye is applied alone or when the two are applied successively.

Neutral red, as has been noted by Hirschler and Monné ('28), has to be rather carefully controlled in its action. I have found that in Gerris the developing acrosome is the first structure to be tinted by it. In the spermatids the dye next stains material inside the acroblast that I consider to be pro-acrosomic substance. With this same degree of staining, a diffuse red area closely associated with the Golgi bodies becomes visible in the spermatocytes. Further staining gives a red color to many granules in the cytoplasm of both spermatocytes and spermatids that were previously visible because of their high refringence (fig. 81). Eventually, the neutral red tinges the general cytoplasm and the chromatin of the nucleus, which is generally interpreted as evidence of antemortem changes in the cell. Practically the only new thing demonstrated in Gerris by neutral red is part of the pro-acrosomic material, as all the other structures are clearly seen in the unstained preparations. Hirschler and Monné ('28) are of the opinion that neutral-red-staining granules inside a Golgi body are artifacts caused by the action of the dye itself and that only the diffuse red area in connection with the spermatocyte Golgi bodies and the acroblast is a normal part of the Golgi complex. In Gerris I have seen this diffuse area break up into bright red granules upon prolonged staining, just as Hirschler and Monné have described. But the spheres of pro-acrosomic material inside the acroblast of Gerris are certainly much different structures. They are demonstrated by a much shorter staining period and are never preceded by a primary diffuse staining of the contents of the acroblast. Although the pro-acrosomic material is not visible before the application of neutral red, it is probably present in essentially the same form as when stained, but invisible, perhaps because it consists of vacuoles of nearly the same refractive index as the surrounding substance.

THE STRUCTURE OF THE TESTIS

All the species of Gerris I have examined have two testes, each of which has two distinct, cylindrical lobes. The lobes have short ducts leading into a common tube which is bent upon itself twice, finally joining the similar structure from the opposite testis. During some part of its course each of the common ducts is dilated to form a seminal vesicle. Text figure 1 shows the testes and ducts of two species of Gerris. The chief specific differences beyond size are in the relation of the seminal vesicle to the loops of the ducts.

The testes of the adult males contain only spermatids and developing sperms, but the middle and late instars have an abundance of spermatocyte stages. Specimens of Gerris marginatus of all ages were collected from June to November. Gerris remigis, however, has but one brood a year, which matures in the month of July, so that only mature forms are obtainable after August 1st.

The testicular lobes show no such marked seriation as is characteristic of those of many Hemiptera. There seems, however, to be a general rule that all the earlier stages are on one side of the lobe (apparently that farthest from the duct), with the spermatogonia at the extreme edge of this zone.

The wall of each cyst is composed of one to three large, thin cells, each of which has a huge, oval nucleus. This epithelial investment retains its relation to the cells enclosed within it, finally forming a sheath around the compact bundles of mature spermatozoa. The sheaths are evidently lost as the spermatozoa leave the testis, since no traces of them are found in the seminal vesicles.

The cells of Gerris remigis are considerably larger than those of Gerris marginatus.

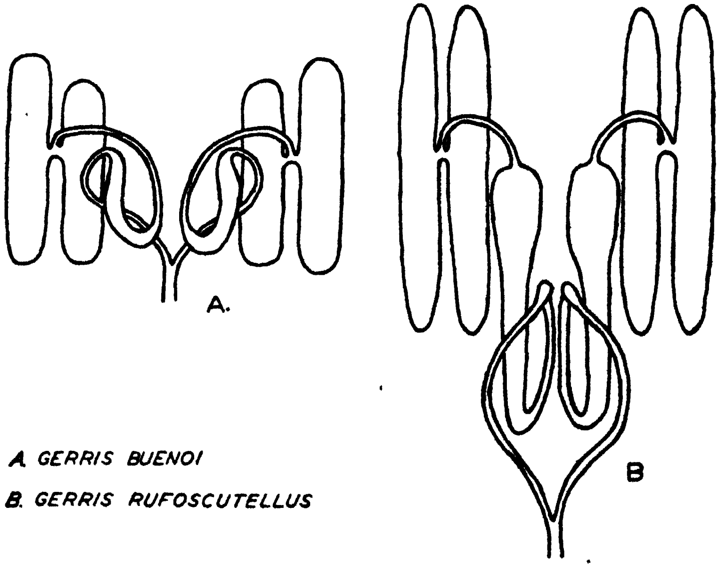


Figure 1

OBSERVATIONS

A. The history of the chondriosomes

The earlier spermatogonia of Gerris are arranged in a rosette group like that described in many other Hemiptera. Such cells have small granular chondriosomes aggregated in a clump at the pole of the cell where the spindle remnant and most of the cytoplasm are found. Figure 1 shows two sister spermatogonia at this stage. The chondriosomes are the smaller, more numerous granules.

In the earliest spermatocytes studied, the chondriosomes have the same shape and distribution as in the spermatogonia (fig. 2). Shortly after the growth period is initiated, the chondriosomes begin to scatter throughout the cytoplasm (figs. 81, 82), during which dispersion they appear to undergo some increase in size. The next change is an elongation of each granule to form a thin, irregularly bent thread, like the typical chondriocont of many tissue cells. The diameter of these threads (fig. 3) is at first much less than that of the granules, but as the growth of the spermatocyte continues, each thread becomes thickened and elongated (fig. 4). This growth proceeds until the cytoplasm contains numerous coarse threads or rodlets (fig. 5).

The description so far applies equally well to either species of Gerris I have studied, although in Gerris marginatus the threads never reach so large a size as those in figure 5. The threads are characteristic of the first maturation division in this species. In the later growth stages of Gerris remigis the larger chondriosome threads develop a series of beadlike swellings, which subsequently become separated to form spherical bodies or chondriospheres (fig. 6). Apparently only the largest threads are involved in this process. Sometimes the spheres are formed first at one end of the thread. This breaking up continues until there are very few, or no, filaments left in the cell. The spheres are not of uniform size, but their average diameter is greater than that of the threads from which they were derived.

The earlier spermatocyte threads show no special distribution or orientation. The centrioles are small, inconspicuous granules, which later assume positions at opposite sides of the cell. They are just inside the cell wall, which is drawn in slightly at the nearest points, and are surrounded by small lightly staining regions free from chondriosomes. As the threads are breaking up into chondriospheres the clear area around each centriole enlarges, pushing the chondriosomes out of that region of the cell. This process is accompanied by changes in the shape and distribution of the chondriospheres in the vicinity of the centrioles by which the spheres become elongated to form short cylinders, the long axes of which are radial to the centrioles. At first only a few of the chondriospheres nearest the centers are involved, but as the size of the asters increases, more and more of the spheres become cylindrical and radially oriented. By the late prophase of the first division the only spherical chondriosomes are a few in the future equatorial region of the first division figure. This orientation of the short cylinders is most obvious in a polar view such as that in figure 7. In Gerris marginatus chondriosomes now have the form of filaments, whose orientation consists merely of a straightening of the filaments (fig. 83).

A lateral view of the first division metaphase (fig. 8) shows that the chondriosomes form two hollow truncated cones with their bases together at the equator and their apices facing the centrioles at opposite ends of the cell. The space thus enclosed is occupied by the spindle. This orientation is unchanged during the anaphase of the first division (figs. 9 and 10).

As the two centrioles of the secondary spermatocyte move apart the cylindrical chondriosomes of Gerris remigis are reoriented so that their long axes continue to point to the nearest centriole. Thus, coincident with the centriole migration the cone of chondriosomes is split into two half cones. Figures 11, 12, 13, and 15 show different stages in this interkinetic reorientation.

During the reorientation of the centrioles and chondriosomes the position of the plate of chromosomes remains as it was at the end of the anaphase of the first division (figs. 10, 13, and 14). After the reorientation of the other structures in the cell is completed, the chromosome plate rotates as a whole to assume its proper position for the second division (figs. 15 and 17).

Obviously, the movements described above should result in a secondary spermatocyte in which the chondriosomes do not form a complete sheath around the spindle, but surround only one side of it (figs. 15, 16, and 17). Figures 15 and 16 should be compared with figures 8 and 7, respectively. During the second maturation division there seems to be no further change in the position of the chondriosomes (fig. 18) and each spermatid receives a half cone of them (fig. 19).

Following this division, there is an apparent migration of the centriole along the periphery of each cell so that those of two sister spermatids approach each other. The chondriosomes at the same time move so that their long axes are continually radial to the centriole, while their distance from it remains constant. As a result of this, when the reorientation is completed, the chondriosomes in each spermatid have the same relation to the centriole as at the end of the second division (fig. 20, an intermediate stage in this process). During this reorientation the chromosome plates undergo a rotation of 90°, so that at the completion of the rearrangement the topographical relationships of the centriole, chondriosomes, and chromosome plate in each spermatid are the same as at the telophase of the second division, although the axis has moved through an arc of 90°. Figure 21 is a lateral view of two sister spermatids, in which the reorientation is completed. This figure should be compared with figure 19.

During the late prophase of the first maturation division of Gerris remigis suitable staining demonstrates that each chondriosome consists of a chromophilic shell and a chromophobic core. This is not evident in Gerris marginatus until the metaphase of the second division, when the filaments become swollen to form short cylinders. The existence of these two substances in the chondriosomes is significant because of the early nebenkern patterns.

I have studied the stages in condensation of the chondriosomes to form a nebenkern and the subsequent transformations chiefly in Gerris marginatus, since in this species crystal violet stains these stages very well. Shortly after the telokinetic movements of the spermatid components, the chondriosomes show a tendency to clump together, forming a more or less continuous ring (figs. 22 and 23). A lateral view (fig. 24) shows that this ring is concavoconvex. As the aggregation of the chondriosomes becomes closer the shape of the mass becomes a thick disc with a central perforation, through which the remnant of the spindle passes (figs. 25, 26, and 84). In the cell drawn in figure 26 the nucleus could be seen by focusing upward. There are probably several layers of chondriosomes superimposed, as shown by the thickness of the disc (fig. 25). I have been unable to find cells in which the individual chondriosomes are distinguishable when the mass is viewed laterally, which is not surprising, since none of the cylinders would be seen directly on end and many of them would be overlapped by other cylinders.

I am not certain of the events immediately subsequent to the thick-disc stage. Probably, coincident with a loss of the influence of the centriole, each cylinder tends to resume its spherical shape. This occurs in Gelastocoris (Payne, '27), where the fusion of the chondriosomes is delayed for a longer time. The spindle remnant probably disintegrates about this time, which may influence the shape of the chondriosome mass. At any rate, the next clearly distinguishable stage finds the chondriosomes in a spherical mass, to be spoken of henceforward as the nebenkern. Focusing at different levels on this sphere demonstrates that it consists of short cylindrical bodies like those shown around its periphery (fig. 27). This figure depicts only the surface of the nebenkern. The elements of the nebenkern next partially fuse, so that their number is reduced without change in their essential character (i.e., a chromophilic periphery around a chromophobic core) (fig. 28). These larger elements apparently continue to run together and to become arranged in concentric shells. An intermediate stage in this process is seen in figure 29, an optical section through the center of the nebenkern. The final result of this fusion is a nebenkern consisting of several curved shells of chromophilic substance fitting inside one another, the spaces between the shells being a continuous mass of chromophobic material (fig. 30).

In Gerris remigis the details are practically the same. In both forms all the stages subsequent to figure 27 are visible in the fresh preparations, as figured also by Lewis and Robertson ('16) in Chorthippus. Prior to that stage no differentiation into chromophilic and chromophobic substances can be distinguished. All the later nebenkern transformations are also visible in the fresh preparations (figs. 87, 88, 89, 92).

The later changes in the nebenkern of Gerris are so similar to those described in detail by Bowen ('22) in the Pentatomidae that a brief summary of the important events is sufficient. There appears within the chromophobe substance of the nebenkern at the concentric-shell stage a new substance in the form of small droplets. This material, which has been called the central substance by Bowen, is very erratic in its staining reactions, occasionally blackening with osmic acid or staining with crystal violet, acid fuchsin, or hematoxylin. More frequently it is unstained, but in all cases it is visible because of a marked difference in refractive index. This last property also enables one to distinguish it in the fresh preparations. The new substance appears first at the periphery of the nebenkern (fig. 31) and progressively develops toward the center of the mass. Accompanying this process there is an apparent dissolution of the chromophilic platework (fig. 32). After the disappearance of the chromophilic material, the nebenkern consists of approximately equal hemispheres. Apparently the chromophilic platework itself is never so divided. The halves of the nebenkern elongate and twist about each other to form two long ribbons with the droplets of central substance in a single row in each of them (fig. 35). This stage is attained somewhat differently in Gerris remigis and Gerris marginatus. In the former the beaded ribbons seem to become spun out from the posterior ends of the main masses (fig. 34), while in the latter the entire nebenkern halves are drawn out (fig. 33). The material in each ribbon next runs together at intervals to form swollen nodes, separated by thin internodes (fig. 36). Finally, the nodes also are drawn out into two spirally twisted threads extending nearly the entire length of the sperm tail (figs. 92, 93). In the nearly mature spermatid this portion is distinctly wavy, probably because of the twisted threads. It is stainable with Janus green in the freshly teased preparations.

B. The history of the Golgi apparatus

I have not been successful with osmic-acid impregnations of the Golgi apparatus in Gerris marginatus, and hence the account will be largely drawn from observations of Gerris remigis.

The youngest spermatocytes I have studied contain more or less spherical Golgi bodies, five to ten in number. They are not aggregated at one pole, but are scattered at random around the periphery of the nucleus (fig. 37). At a slightly later stage (fig. 38) the shape has changed from spherical to cylindrical. Both these stages are from acid-fuchsin preparations of Gerris marginatus and no differentiation within the Golgi bodies can be made out. Their identification rests on the fact that on these slides only Golgi material is stained with the acid fuchsin.

The Golgi bodies next increase in number (fig. 39). The occurrence of several groups of two Golgi bodies suggests that this increase may be by splitting of the original bodies. The Golgi bodies are now composed of an osmiophilic shell enclosing non-osmiophilic contents. The increase in number continues until there are from fifteen to twenty Golgi bodies in each cell. As the different views of them given in figure 41 show, they are ovoid bodies with the differentiation mentioned above. Following the primary multiplication, each body increases considerably in size and changes in form. The spheres first become altered to the form of double-walled cups, apparently by the infolding of one side (figs. 41, 42, 43, and 44). In these cups the double wall is formed by the osmiophilic material, and the non-osmiophilic material of the earlier Golgi bodies occupies the space between the two walls. The thickness between the walls varies. Sometimes the non-osmiophilic material is almost completely obliterated by the apposition of the walls (fig. 44). The reduction of the number of Golgi bodies in figures 42 and 43 is only apparent, being due to the increase in size of the cells so that a section includes a smaller part of the whole cell.

As the increase in size of the Golgi bodies continues the osmiophilic material becomes folded and wrinkled very irregularly, so that their shape cannot be ascribed to any general pattern. Figure 45 is a drawing of a cell in the late growth period when the Golgi bodies have reached their maximum size without change in number. They consist of a much wrinkled osmiophilic envelope, but whether this encloses a non-osmiophilic material cannot be determined in fixed preparations. The number of Golgi bodies is still fifteen to twenty.

During the prophase of the first maturation division two changes are occurring in the Golgi bodies. They are becoming arranged in a belt in the region of the equator of the first division figure, and they are also fusing together to form several (usually three to five) large masses (figs. 46 and 47). It seems to me probable that their fusion is incidental to their accumulation at the equator, which brings the separate bodies into contact. Their general structure seems to be very much like that of the individual bodies prior to fusion. This prophasic fusion seems to have little significance, as the masses soon begin to break up into small, irregular fragments (fig. 48). Before the anaphase of the first division the Golgi material is entirely in the form of these scattered fragments (dictyosomes). These are distributed rather uniformly throughout the cytoplasm, although there appears to be a special accumulation of them at each pole in the form of a ring around the aster (fig. 49).

The dictyosomes maintain their general distribution unchanged throughout the succeeding division stages (fig. 50), and are thus apportioned in approximately equal number to the two secondary spermatocytes. I have not been able to make out the interkinetic redistribution, but the second division metaphase shows the same distribution as in the first division figure (fig. 51).

During the early stages in the fusion of the chrondriosomes the dictyosomes remain scattered uniformly throughout the spermatids (fig. 52). With the assumption of a spherical shape by the nebenkern, the dictyosomes begin to fuse together, forming at first a number of shells somewhat like those of the early spermatocytes (fig. 53). The fusion progresses (figs. 54, 55, and 57) until the Golgi material consists of a single large aggregate lying in the angle between the nucleus and the nebenkern (fig. 56). This structure, because of its relation to the development of the acrosome, is frequently called the acroblast.

The figures of the acroblast and of the larger spermatocyte Golgi masses do not give a clear idea of their structure, because they are drawn as single optical sections. This fact makes the osmiophilic material appear as lines or threads (figs. 47 and 58). However, focusing shows that the acroblast is a much wrinkled sac of osmiophilic material, which is widely open at one point, where the acrosome is developed.

In the fresh, unstained preparations of the testes of Gerris the Golgi apparatus in the later spermatocytes and spermatids appears as a light network (*AB*, fig. 85). This network is very suggestive of the dark osmiophilic lines seen in the osmic preparations. Focusing shows that this light network is really an optical section of a wrinkled sac. The spherical acrosome is attached to one side of the acroblast. This acrosome stains in fresh cells with neutral red. The dye also demonstrates inside the acroblast smaller spheres of various sizes (figs. 86 to 90), which are the pro-acrosomic material.

In the spermatocytes examined in the fresh condition the peripheral osmiophilic Golgi material can be seen only in the prophase of the first division, when the Golgi substance is in the form of large masses in the equatorial region of the cell (fig. 83). The contents of these masses stain with neutral red. After the fragmentation of the Golgi masses to form dictyosomes, these red-staining areas are still seen in the equatorial region, showing that only the peripheral material is involved in the fragmentation and distribution throughout the cell. In the secondary spermatocytes and very early spermatids the red areas are no longer visible, and in fact, nothing in the cell stains with neutral red, until in the later stages of its action it tints refringent granules as described under Methods. In earlier spermatocytes neutral-red-staining areas are seen scattered throughout the cell in about the same number as the Golgi bodies, but I have been unable to see the light material corresponding to the osmiophilic substance around these spheres.

The earliest appearance of the acrosome results from fusion of several smaller masses that appear during the later stages of fusion of dictyosomes to form the acroblast (figs. 57 and 58). The acrosome increases enormously in size throughout the period when the nebenkern transformations are taking place, apparently as a result of addition to the main acrosomic mass of the pro-acrosomic material that appears inside the acroblast (figs. 86 to 91). When the growth of the acrosome is complete, no more spheres are formed inside the acroblast (fig. 92). After the acroblast has separated from the acrosome, no neutral-red-staining material is associated with it (fig. 93).

By what one might call a type of microdissection I have been able to determine something of the physical nature of the acroblast and its relation to the acrosome and pro-acrosomic material. The 2-mm. water-immersion lens that I have used to study fresh mounts is used so close to the coverslip that it is possible to focus down enough to crush the cell and burst its membrane, and yet to keep the cell in approximate focus. If this is carefully done none of the cell components are crushed enough to be damaged. The rupture of the cell membrane causes the immediate dissolution of the general cytoplasm and nucleus. The nebenkern very quickly swells and disappears, and in a very short time everything has disintegrated except the acroblast and its associated structures. These neither swell nor shrink, but remain in the Ringer's fluid apparently unaltered until crushed by pressure due to evaporation at the edge of the coverslip. They may persist thus for as long as thirty minutes. The attachment between acrosome and acroblast persists during all this time, and the pro-acrosomic spheres inside the acroblast are unaltered. Such an isolated complex of acroblast and acrosome is drawn in figure 90. The acrosome may be distorted, but regains its spherical shape as soon as the distorting force is removed. Apparently the acroblast of Gerris is a fairly rigid envelope, and it is this envelope which is demonstrated in fixed preparations, because of its tendency to blacken with osmic acid.

C. The later differentiation of the acrosome

During the early stages of its growth the acrosome appears homogeneous except for a granule on the side toward the nucleus (*AC*, figs. 33, 59, 60, 61, and 90). This granule stains with hematoxylin and sometimes with neutral red. The later differentiations are shown best in the fresh preparations or in Weigl preparations (in hematoxylin, Kull, and Benda preparations the entire acrosome is usually heavily and uniformly stained). In the middle growth period the acrosome is divided into two substances, which, when fixed, appear of different refringence (*AC*, fig. 62). Later, one of these becomes granular and the other remains homogeneous (figs. 63 and 91). The latter is directed toward the nucleus, but I cannot say whether it has any relation to the granule previously seen. In the later stages of growth both these regions enlarge until the acrosome is an enormous structure that occupies the major part of the spermatid (*AC*, figs. 64 and 92). Along one side of this mass is the acroblast (*AB*), now relatively very small, but still in intimate contact with the acrosome.

As the acroblast separates from the acrosome the latter loses its affinity for neutral red. It consists at this time of a highly refringent crescent (*CA*) surrounding a sphere (*SA*), less sharply defined (figs. 72, 73, and 93). In fixed preparations, and occasionally in fresh preparations, it is evident that the crescent consists of a main body, a tip, and two large granules at the proximal end. These granules are both clearly visible in an oblique view (fig. 73), but in a view lateral to the crescent the two granules are in line with the plane of observation (fig. 72). As soon as the mass of residual protoplasm with the acroblast has moved out of the sperm head, the final stages in acrosomal differentiation begin. The crescent straightens out to form a short, lance-shaped structure (*CA*) in contact with the sphere (*SA*) at one side (fig. 74). The lance-shaped structure elongates, and as it does so the sphere that is in contact with it is stretched out along it to form a partial sheath. This is shown in figure 75, where the sheath (*SA*) is stained more heavily. With most dyes the sheath stains like the core of the acrosome so that the relation between the granules and tip of the latter can be clearly seen, but the core and the sheath of the acrosome (*AC*) cannot be distinguished. In a view that shows both the large granules it is seen that there is a smaller granule between them (fig. 76). The large granule and the differentiated tip of the acrosome at this stage are always clearly visible in the fresh preparations. In fixed material the granules and the tip have a strong affinity for crystal violet.

The acrosome becomes very much elongated until its diameter is no greater than that of the tail of the spermatid. During the early stages of its elongation its main components can still be made out (fig. 78). Shortly after this stage the granules elongate to form rods parallel to the long axis of the acrosome. In the later stages the only signs of differentiation are a single granule at the base of the acrosome and the differentiated tip (figs. 79 and 80). Examination of the mature sperm shows that the acrosome forms practically half of its length, becoming so attenuated that it is barely visible with a magnification of 400 ×.

D. The 'chromatoid body'

In the later spermatocytes of both Gerris remigis and Gerris marginatus there are one or two chromophilic bodies that are apparently related to none of the other cytoplasmic structures. These bodies are variable in size and occurrence. They are usually curved rods, somewhat sickle-shaped (*C*, fig. 66). They are distinguished technically by staining with alizarin by the Benda method and staining with acid fuchsin in the Kull preparations. They also have an affinity for hematoxylin after all methods of fixation and mordanting. These bodies are not visible in fresh preparations.

The behavior of the bodies during the maturation divisions is the chief point of interest. They are apparently oriented at random in the cell and behave according to their chance relation to the plane of cleavage. Figures 67 and 68 are examples of a second division metaphase and telophase, respectively, in which the body had gone entirely into one cell because of the orientation it had during the prophase and metaphase. Figure 69 shows a division figure in which one body has gone entirely into one of the spermatids, while another is being divided because of its position at the equator. In the spermatids these bodies persist for some time, apparently unchanged (*C*, figs. 26, 27, 28, and 70). Perhaps some, if not all, of the chromophilic granules found in the later spermatids have resulted from the fragmentation of this substance (*C*, fig. 33).

These chromophilic bodies have some of the staining reactions described by other observers for the chromatoid body. Their behavior is somewhat different in that they are sometimes divided. On the whole, however, I am inclined to believe that the designation, 'chromatoid body,' is indefinite enough to describe them as accurately as the information warrants.

E. The end bulb of the axial filament

It is perhaps worthy of note that as the axial filament (*AF*) grows out from the spermatid of Gerris it carries a little bulb of homogeneous material on its tip (figs. 32, 70, 86, and 87). A similar structure has been described by Gatenby ('17) in the Lepidoptera. It appears to persist at least to the beaded ribbon stage of nebenkern elongation (fig. 71), but it is certainly not present in the late spermatids, unless very much attenuated.

F. The centrioles

Gerris does not seem to be a favorable object for the study of the centrioles; at least, I have not been able to demonstrate them during the critical spermatid stages. The only item of possible interest is the fact that in fresh preparations of the spermatocyte divisions I have seen two highly refringent dots in the center of each clear polar area. These are almost certainly the centrioles. The centrioles are also clearly visible in the early spermatids (*CE*, fig. 84). I mention these results because I am not aware of any other observations of centrioles in spermatocytes in fresh preparations. Doubtless they would have escaped my notice here, had their positions not been clearly marked by the radial orientation of the chondriosomes.

G. The mature spermatozoon

The elongation of the spermatids continues for some time after it has become impossible to recognize the differentiated elements. In fact, when the nebenkern halves are still beaded ribbons the length is so great that a section of 8 μ includes only a small section of a spermatid. For this reason the size and shape of the older spermatids and mature spermatozoa can only be studied in teased preparations.

When the testis of a mature animal is teased apart, the later spermatids are separated out in bundles bound together by the large follicle cells. In the earlier stages these bundles have a bulb at one end, where the main cell bodies are situated. Later the diameter of the bundle becomes more nearly uniform as the acrosome and nucleus become elongated to the approximate diameter of the posterior part of the spermatid. The later spermatids in Gerris marginatus are differentiated into two approximately equal regions, one untwisted and another that is uniformly waved along its entire length. The wavy portion stains with Janus green, and if the spermatozoa are nearly mature, this portion will begin active movement soon after the bundle is broken apart. This part almost certainly is formed from the axial filament and the twisted chondriosomal threads. The wavy portion and a short adjacent unwaved portion are of greater diameter than the remainder of the spermatid. In the oldest spermatids (sperms) of Gerris marginatus the length of the wavy portion was approximately 1.5 mm.; of the shorter unwaved part, 0.2 mm., and of the longer unwaved region, 1.5 mm. The total length, then, is over 3.0 mm. From these proportions I think it probable that the thicker straight part represents the elongated nucleus and the thinner straight part the acrosome. It is perhaps noteworthy that the mature egg of this species is about 0.8 mm. in length, slightly more than one-fourth that of the spermatozoon.

I have teased out mature, active spermatozoa from the seminal vesicle of Gerris remigis. The activity is always confined to the tail region. Their total length is approximately 5.0 mm., with the proportions similar to those of the spermatids of Gerris marginatus. These mature spermatozoa do not occur in bundles and no trace of the cells forming the sheaths of the bundles in the testis can be found in the vesicle. The wavy portion of the tail is very short, the majority of it being unwaved. The acrosome is elongated so much that for a large part of its length it is barely visible with a magnification of 400 diameters. Spermatozoa teased out from the vesicle are very active. The tail portions are constantly writhing, winding and unwinding upon themselves, and in many cases apparently becoming inextricably tangled with one another, especially in the dilated part of the seminal vesicle. In the narrower distal part of the vesicle they are somewhat less tangled, but they are by no means so well oriented as to make it easy to understand how their passage to the female and the subsequent process of fertilization can take place. The egg of Gerris remigis is 1.0 mm. in length.

DISCUSSION

A. Introduction

The Hemiptera-Heteroptera have long been a favorite group for studying various aspects of spermatogenesis. The most complete accounts of these phenomena that we have, both from the standpoint of nucleus and of cytoplasm, are those for various members of the family Pentatomidae, due to the work of Montgomery on Euschistus and later of Bowen on numerous members of this family. The present study shows the spermatogenesis of the Gerridae to be remarkably like that in the Pentatomidae. As far as the cytoplasmic components are concerned, there is the same relatively great increase in mass during the growth period; at the maturation divisions both Golgi material and chondriosomes are partitioned in approximately equal parts to the four spermatids, where the chondriosomes become aggregated to form the nebenkern and the Golgi material to form the acroblast. In spermiogenesis the nebenkern becomes eventually drawn out into two spiral threads in the tail of the spermatozoon by a complicated series of changes. The acroblast of the spermatid, after having been intimately concerned in the production of the acrosome, passes down the spermatid tail and is sloughed off with a mass of residual protoplasm.

Such a general description could be applied with equal accuracy to the phenomena in Gerris or to those described in Euschistus, Murgantia, and other Pentatomidae by Bowen. The present results, therefore, are significant for the general phenomena of spermatogenesis only in a confirmatory sense. Accordingly, the discussion will be confined to special details that seem to be unique or unusually emphasized in Gerris.

B. The chondriosomes

The transformation of granular chondriosomes into the filamentous type has been frequently described. It has been followed in living cells cultivated in vitro by Lewis and Lewis ('15). It has been described in the spermatocytes of Cicada

(Schaffer, '20), of Fasciolaria (Hyman, '23) of scorpions (Wilson, '16 and '25, and Gatenby and Battacharya, '25), of Saccocirrus (Gatenby, '22), and of the Belostomatidae (Chickering, '27). Opinions differ as to the exact method by which the transformation occurs. In Fasciolaria, Hyman clearly shows that the filaments arise by linear fusion of the separate granules. On the other hand, Lewis and Lewis state that this phenomenon is rarely observed in tissue cultures, fusion of granules commonly producing larger granules. They usually observe the formation of filaments by the drawing out of granules. Chickering concluded that the Belostomatidae show the same process as in Fasciolaria, but his figure of the intermediate stage is not very convincing. Chickering's argument, from analogy with Gryllotalpâ (Voinov, '25) and the Lepidoptera (Gatenby, '17), does not seem to me sound, because in these cases the chondriosomes are very large and probably of the type of the chondriospheres of Gerris rather than the granules of the early spermatocytes and spermatogonia. In Gerris the much smaller diameter of the filaments and the fact that their number seems to be little, if any, less than that of the granules make it almost certain that the process is like that described by the Lewises.

The orientation of the chondriosomes with respect to the centrioles is a most striking characteristic of the maturation divisions of Gerris. An orientation of filamentous chondriosomes similar to that described in Gerris marginatus has been previously observed in Apis (Meves, '07), in the Pentatomidae (Bowen, '20), and in Stenobothrus (Bělăr, '27). In Gelastocoris, Payne ('27) has described an orientation of spherical chondriosomes involving a change to a cylindrical shape like the case in Gerris remigis. Other more special examples are those in the dividing egg of Echinarachnius (Hibbard, '22) and in the spermatocytes of Gryllotalpa (Voinov, '25). The chondriosomes in the former case are small granules, which Hibbard observed to become aligned in rows radial to the center of the asters. In Gryllotalpa the chondriosomes at the metaphase of the first division are long cylinders that form a palisade around the spindle. Each cylinder extends the entire length of the spindle and is swollen considerably at the equator. The ends of the chondriosomes are drawn out toward the centers much as the spheres in Gerris remigis and in Gelastocoris are elongated to form cylinders.

Probably the diversity in behavior of the chondriosomes in division in these examples is mainly due to differences in the morphology of the chondriosomes before they came under the influence of some factor localized at the poles of the cells. Hibbard has suggested that the orientation in Echinarachnius is due to currents radial to the centrioles. This explanation appears adequate to explain many of the cases described in animal cells. The currents which Hibbard refers to appear to be identical with those described by Chambers ('23) as flowing toward the centers of the asters, and which when fixed give the appearance commonly described as astral rays. If this is the sole force responsible for the chondriosome orientation, one should expect the arrangement to be most marked when the asters are very highly developed, as they are in echinoderm eggs, for instance. This expectation meets with quite formidable obstacles, especially in the case of the higher plants, where, in spite of the absence of asters, there is frequently a polar orientation of cytoplasmic elements (notably the archiplasts) that is more striking than in most animal cells. Furthermore, I have seen the opposite exception in animal cells in the case of the spermatocytes of Notonecta. In these cells the asters are unusually large and clearly defined, and yet, as far as my uncompleted observations go, they indicate that orientation of cytoplasmic elements radial to these asters is very slight, if not completely lacking.

The interkinetic phenomena are chiefly of interest as emphasizing how the cytoplasmic orientation is apparently regulated by the centrioles. Similar facts have been briefly described in Murgantia (Bowen, '20). Whatever we consider the nature or function of the centrioles, as far as can be seen in the spermatocytes of Gerris, they are the focal centers for the organizing and regulating of the rearrangement of the whole cell involved in preparation for a second maturation division. The asymmetry of the chondriosome distribution in the secondary spermatocyte indicates clearly that, with the exception discussed below, there are no extensive movements in the cytoplasm other than those that can be definitely correlated with the separation of the focal centers.

One interesting feature of the interkinetic stages is the fact that the chondriosomes and centrioles are entirely reoriented before the chromosome plate rotates into the equatorial plane of the second division figure. Probably up to this time the chromosome plate is held in position by the remains of the first division spindle, which is still present at a stage like that of figure 12, as a cylindrical region free of chondriosomes. The spindle has been shown by Chambers ('23), Bělăr ('27), and others to be an area that is considerably denser than the surrounding cytoplasm. Hence its continued existence would apparently account for the fact that the chromosomes, of all the visible cell structures, are not reoriented concomitantly with the separation of the centrioles. The chromosomes are finally arranged on the second maturation division spindle not primarily because the centrioles have reached positions at opposite ends of the cell, but more probably because, coincident with their assumption of that position, there is a degeneration of the remains of the spindle of the first maturation division. The chromosomes then are free to be acted upon by whatever force is responsible for their assuming the equatorial position in any dividing cell.

To summarize the above briefly: a consideration of the maturation divisions of Gerris primarily from a standpoint of cytoplasmic orientation shows that the forces responsible for the symmetry of the dividing cell are definitely localized in the region of the centrioles. The bipolarity that has long been evident with respect to chromosomes, spindle, and asters is also clearly shown by the orientation of the cytoplasmic components.

The nebenkern changes subsequent to the stage of figure 22 have been thoroughly worked out in the Pentatomidae (Bowen, '22). In Gerris the facts are almost exactly the same. The most significant difference is in the time when the differentiation of the chondriosomes into chromophilic and chromophobic substances is first observed. In the Pentatomidae this becomes evident only as the chondriosomes are condensing to form the nebenkern sphere. In the Lepidoptera (Gatenby, '17 a, and Bowen, '22) and in *Helix* (Gatenby, 17 b), on the other hand, the chondriosomes show this differentiation in the spermatocytes. The two species of Gerris are intermediate between these two types, the differentiation in one species appearing in the prophase of the first division, while in the other it is not evident until the second division.

C. The structure of the Golgi apparatus

The structures described as the 'internal reticular apparatus' and later as the Golgi apparatus are of highly variable characteristics in different tissues. They are of relatively complicated form as compared with chondriosomes and chromosomes, and are demonstrable only by highly capricious techniques. Earlier opinions apparently tacitly agreed, largely on the basis of studies of the Golgi apparatus in somatic tissues, that, whatever might be its exact morphology, it probably represented a homogeneous material. Furthermore, these earlier descriptions indicated that the Golgi apparatus had the character of a continuous or discontinuous network. The general description to be found in most modern text-books comprises the two above features.

As a result of his studies on the molluscan embryo and larva, Hirschler ('18) described the osmiophilic Golgi body as a hollow sphere. Although hemispheres were frequently observed, Hirschler considered that these were artifacts. He pointed out that this hollow sphere, the Apparathülle, separated the contents of the sphere, the Apparatinhalt, from the surrounding cytoplasm, and suggested that the rôle of the Golgi apparatus was that of an isolation membrane. In this paper Hirschler does not emphasize the possibility of the Apparatinhalt being a specific material different from the surrounding cytoplasm. In some tissues of the molluscan larva Hirschler found that the spheres became irregular lamellar bodies. These structures in some cases became contiguous, forming an irregular lamellated structure, which in optical section resembled the classical descriptions of the reticular apparatus.

In a later paper ('27), Hirschler again emphasizes the fact that the osmiophilic material is essentially lamellar in structure, and he lists an imposing array of descriptions of such a type from many different authors. He appears to have modified his earlier viewpoint to the extent of considering the apparatus as composed of either closed spheres or open hemispheres.

In 1920, Bowen, as a result of his study of the Golgi apparatus in the spermatocytes of the Pentatomidae, concluded that it had a duplex structure, consisting of an osmiophilic component (corresponding more or less to the classical Golgi apparatus) and a non-osmiophilic component (previously described as the idiosomic substance). Bowen tentatively concluded that the Golgi apparatus in general had this structure, but he did not generalize as to the topographical relationship of the two components.

In his 1927 paper, Hirschler has modified his conception of the structure of the Golgi apparatus so that it seems to be nearly identical with that of Bowen. He considers the Apparathülle to be identical with Bowen's osmiophilic substance and the Apparatinhalt to correspond to the idiosomic substance. He is no longer insistent upon a uniform topographical relationship between the two components.

Nearly all the work on the Golgi apparatus in spermatocytes has demonstrated what appears to be a duplex structure, although the topographical relationship between the two components may vary widely in different forms. Commonly the Golgi material consists of numerous spherical or hemispherical bodies, the surface of which is osmiophilic—an arrangement which has been termed polyvesicular by Hirschler. This has been demonstrated in the spermatocytes of the Coleoptera (Bowen, '22), Peripatus (Gatenby, '25), Notonecta (Voinov, '27), Gelastocoris (Payne, '27), and Pollia (Tuzet, '28). In the Pentatomidae, Bowen described Golgi bodies in the form of discs, which, in profile, often appeared to be paired rods. The osmiophilic material formed two concentric crescents around one side of this disc, the remainder of its surface being the non-osmiophilic idiosomic substance. Essentially the same structure has recently been described in the Belostomatidae (Chickering, '27). It is not clear what is the relationship between these discs and the more usual spherical or hemispherical Golgi bodies. That they are some variation of the usual form is indicated by Chickering's observation of an abnormal specimen in which the Golgi bodies were actually spherical instead of discoid. A Golgi apparatus consisting of a single mass with osmiophilic periphery (Hirschler's univesicular type) has been described in the Pseudoscorpionidae (Sokolov, '26) and in Tegenaria (Sokolska, '24). In many forms there has been described a condition where the Golgi bodies consist of a number of disconnected crescents or concave discs on the periphery of an idiosomic mass. The early observations of Murray ('98) on the 'nebenkern' of Helix and those of Meves ('96) on Salamandra, as well as the most recent results of Hirschler and his students on fresh preparations, all indicate that in most cases these separate Golgi bodies are actually optical sections of a somewhat wrinkled envelope completely surrounding the idiosomic mass. Hence these probably may be assumed in many cases to fall into the univesicular group. Examples of this structure have been described in the Gasteropoda (Murray, '98; Monné, '27), Mammalia (Sjövall, '06; Hirschler and Monné, '28), Pisania (Tuzet, '27), and Lepidoptera (Hirschler, '28 a and b). Possibly it is the same in Palamnaeus (Gatenby and Battacharya, '25), Opisthocanthus (Wilson, '25), and urodele Amphibia (Meves, '97, and Bowen, '22). In Fasciolaria, Hyman ('25) has described the derivation of small vesicular Golgi bodies by a sort of budding from a large vesicle.

Voinov ('25) has questioned the conception of a duplex structure in the Golgi apparatus of the spermatocytes. He believes the Golgi material to be essentially homogeneous, although he admits that it may become temporarily heterogeneous by the elaboration of special products within it (e.g., the acrosome in a spermatid). The work of Hirschler and his students on fresh preparations is of interest in connection with this question. They find that in many cases the Golgi apparatus consists of a peripheral envelope which is visible without staining and which corresponds to the osmiophilic material in the fixed preparations. When neutral red is applied to such a cell, the contents stain a uniform red color, while the envelope and general cytoplasm remain unaffected by the dye. As already described, essentially the same results are obtainable with Gerris.

The Golgi bodies of the spermatocytes of Gerris at all stages I have studied by osmic-acid impregnation belong to the polyvesicular type. The earlier Golgi bodies are simple spheres much like those described in many other forms. From the fixed preparations it is impossible to say whether the growth of the Golgi bodies involves both components or not. Appearances like those in figure 44 would seem to indicate that in many cases the non-osmiophilic contents of the Golgi vesicles become greatly reduced or obliterated by the apposition of the walls. This certainly is not the case if the idiosomic substance is stainable with neutral red, since a diffuse stain is seen in the contents of the masses resulting from the prophasic fusion (fig. 83). The double cup figures like those of figure 43 are not very common, however, and probably they merely indicate an initial stage in the complex folding undergone by the osmiophilic material.

The prophasic fusion of Golgi bodies has, so far as I can find, been described only in Brochymena (Bowen, '20).

The behavior of the Golgi apparatus during maturation division appears to be highly variable. In some cases dictyosomes are formed and distributed to the two daughter cells as in Gerris. This has been described in Pulmonata (Murray, '98), the Pentatomidae (Bowen, '20), Stenobothrus (Ludford and Gatenby, '21), Amphibia (Bowen, '22), Fasciolaria (Hyman, '23), and the Pseudoscorpionidae (Sokolov, '26). In all these examples the dictyosomes are subsequently massed in larger number at the poles of the cell around the asters. In other cases the 'univesicular' Golgi apparatus is described as undergoing binary fission before or during the division. Examples of this are the following: Cyrtophyllus (Morgan, '25), Mus and Cavia (Ludford and Gatenby, '21), Palamnaeus (Gatenby and Battacharya, '25), Opisthocanthus (Wilson, '25), and Pisania (Tuzet, '27). The case of Pisania is rather remarkable, in view of the fact that in Fasciolaria, a nearly related form, Hyman had found formation of dictyosomes. Still different are the results found in Gryllotalpa by Voinov ('25), where the single Golgi mass divides twice in the late growth stages to form four parts. At the first division two of these are at each pole, and at the second division one at each pole, so that each spermatid gets one Golgi body. In cases where the Golgi bodies are small and numerous there may be no fragmentation and merely a random distribution of the bodies, as in Gelastocoris (Payne, '27). In Tegenaria (Sokolska, '24) there is a single large Golgi body which goes to one of the secondary spermatocytes. This is fragmented into dictyosomes at the second division and these are apportioned in approximate equality to the spermatids. In the light of our present knowledge of the behavior of the Golgi bodies during mitosis, there seems, therefore, to be no general explanation that will cover all cases. However, it is significant that in all examples except the anomalous one of Tegenaria the four spermatids get approximately equal amounts of Golgi material.

The question of how the duplex structure of the Golgi apparatus of the spermatocytes is affected by fragmentation to form dictyosomes is largely a matter of conjecture. Apparently in Gerris the red-staining core of the Golgi masses and their osmiophilic peripheral parts separate with the prophasic fragmentation of the latter. Although the red-staining material can be seen in the equatorial region at the metaphase of the first division, it cannot be seen in either the secondary spermatocytes or the early spermatids. Red-staining spheres later appear inside the acroblast, but these stain more heavily and appear to be much different from the material associated with the Golgi masses during the prophase of the first division. These observations indicate that the neutral-red-staining core of the Golgi apparatus of the spermatocytes is probably merely a temporary associate of the peripheral osmiophilic material, perhaps arising as a by-product of some activity of the latter. From my study of Gerris I can find no evidence for a fundamental duplicity of structure of the Golgi apparatus. It is my opinion that a final decision on this question must await the appearance of further evidence, more especially along the line of the relation of the spermatocyte Golgi bodies to the very different structures described under that name in somatic cells.

D. The formation of the acrosome

The observation of Bowen ('20) that the acrosome of the pentatomid spermatozoon is synthesized in close relation to the Golgi apparatus (acroblast) has since been shown to be of very wide application. Of many cases studied, this relationship has been found to hold in all but three.

One exception is the condition in Gelastocoris (Payne, '27), in which the acrosome arises from fusion of a large number of small spheres present throughout the later growth period. As the fusion is progressing it can be seen that the larger spherical aggregates have the very small Golgi bodies around their peripheries, and in the huge acrosome that finally results from the fusion the same relation holds. Payne was unable to make out the origin of these pro-acrosomic spheres and saw but vague indications of their possible primary relation to the Golgi bodies.

A similar case has been described in Notonecta (Voinov, '27), in which the acrosome is partly formed from fusion of the elements of the vacuome (i.e., pro-acrosomic material of Payne) and partly by contribution from the Golgi bodies grouped around the acrosomic sphere in the spermatid. Probably in this case, as well as in that of Gelastocoris, we have to do with precocious formation of acrosomic material, in connection with the Golgi bodies when formed, but subsequently freed from them in the spermatocytes. In both examples the Golgi bodies are small and numerous, so that the contribution of each would perhaps not be noticeable at the time of its formation, and would become evident only after it had separated from the body. Voinov found that the 'vacuome' material which is transformed into the acrosome is stainable with neutral red. This fact is also true of the pro-acrosomic material inside the acroblast of Gerris.

The situation in Gryllotalpa is less easily explained. In this form Voinov ('25) concluded that the acrosome arose as a result of the activity of two structures, the acroblast and the 'appareil sphérulaire.' The latter is a cell component apparently having no homologue in any other form. It consists of a number of large spheres which are closely aggregated in typical cases and which persist without much change from the spermatogonia to the early spermatid. Here the elements become aggregated and somewhat fused together. This 'appareil sphérulaire' is found in close association with the acrosome after it has separated from the acroblast, and Voinov accordingly holds the 'appareil' to be a secondary contributor to the acrosome. Voinov's evidence for this conclusion seems to me insufficient and inconclusive. His case is weakened chiefly by his observation that the acrosome continues to impregnate with osmic acid after separating from the acroblast. Such a reaction of the acrosome has not been described by anybody else, so far as I can find. Although many parts of the spermatid may become blackened, I have never seen the acrosome so affected. Probably a small part of the acroblast substance accompanies the acrosome for some time after the main mass has separated from it. The final separation of such a small part of the acroblast might easily have been overlooked by Voinov, and the presence of this element would be enough to account for the slight increase in size of the acrosome after it has separated from the main acroblast without invoking the agency of the 'appareil sphérulaire.' Whatever the broader significance of this structure may be, its topographical relation to the acrosome is probably fortuitous. Certainly the evidence adduced from Gryllotalpa is not strong enough to warrant us in considering it a unique situation without supporting evidence from other examples.

The formation of the acrosome in the animal spermatid presents a very valuable opportunity to study the function of the Golgi apparatus. Although a vast amount of work has been done on the rôles of both Golgi bodies and mitochondria in the formation of secretory products in various types of glandular epithelium, the results in no cases are absolutely conclusive, because of the random and frequently overlapping distributions of these cell components. The work of Morelle ('26) on the formation of secretory granules in the pancreas illustrates the difficulty of such a study. While the general distributions of Golgi apparatus and chondriosomes were found to be distinct in that they were at opposite poles of the cell, it was unfortunately true that there were a few chondriosomes in the exact region of the Golgi apparatus. This fact led Morelle to conclude, somewhat unjustifiably, that the two cell elements play supplementary rôles in the elaboration of secretory granules. In the animal spermatid, however, there is no question of the absolutely distinct distribution of mitochondrial and Golgi substances. The mitochondria are very early aggregated into a compact mass, the nebenkern, while the Golgi material is similarly aggregated into a distinct body, the acroblast. Furthermore, long before the completion of the process of spermiogenesis the main bulk of the mitochondrial material has been spun out into two long threads running down the spermatid tail, while the acroblast is left in the head region until almost the last stages. In studying the process of acrosome formation there can be no question of mitochondria playing any direct rôle. So far as this one process is concerned, our conclusions as to the function of the Golgi apparatus are as conclusive as any based upon observation alone can be.

The rôle of the acroblast in the formation of the acrosome is more clearly demonstrated in Gerris than in any other form studied. The acrosome itself is stainable with neutral red during all the stages of its growth, and this fact serves as a means of identifying pro-acrosomic material. The pro-acrosomic material is present in the form of spheres of various sizes inside the acroblast sac throughout the period when the acrosome is growing in size. After the acrosome has completed its growth, there are no spheres present inside the acroblast and no more are formed inside it after its separation from the acrosome. That all of the final process of acrosome synthesis is going on inside the acroblast is rather strikingly demonstrated by the fact that the acroblast isolated by crushing has the acrosome attached to its edge and numerous pro-acrosomic spheres inside it. One is tempted to describe this as like a factory inside the cell for the elaboration of acrosome material, since it appears to be such a distinct unit. In Gerris it seems highly probable that the acrosomic material is formed primarily as the result of synthetic processes in the interior of the sac of osmiophilic Golgi substance.

The pro-acrosomic spheres and the acrosomic material are probably distinct chemically as well as morphologically. The acrosome itself stains heavily with basic dyes in fixed preparations, while only infrequently can pro-acrosomic material be demonstrated by this method. Usually one or two spheres inside the acroblast stain exactly like the acrosome. After their first origin, when they are not stainable in fixed preparations, the pro-acrosomic spheres probably undergo a gradual change so that they take on an affinity for basic dyes. This is the reaction of the acrosome proper and in this form the pro-acrosomic spheres are added to the main mass.

Complex acrosomes like that of Gerris have been described in several insects, e.g., in Notonecta (Pantel and de Sinety, '06) and Gelastocoris (Payne, '27). These complex acrosomes seem in general to agree in consisting of two main masses, within which there may occur differentiations of various sorts, as in Gerris.

A unique theory of acrosome development is that of Maurice Parat ('28). This author accepts practically all facts about acrosome formation in such forms as Gerris, but interprets them in a manner very different from that of other cytologists. His theory is based upon observations made with vital dyes, chiefly neutral red and Janus green, which he believes to have a high degree of specificity, the former for watery vacuoles and the latter for chondriosomes. Since the early acrosome stains with neutral red, Parat regards this as a vacuole and holds that the mature acrosome is formed by a process of dehydration and condensation within this structure. These vacuoles are regarded as the fundamental cytoplasmic elements in the synthetic process, the accumulation of Golgi material (lepidosomes, according to Parat) in that vicinity being a secondary result of their presence. The facts in Gerris are very definitely opposed to this view and in favor of the conclusion that the Golgi apparatus is the fundamental active cell component, through the agency of which the acrosomic material (in the form of spheres stainable with neutral red) is synthesized. From the later stages of the first maturation division to the formation of the acroblast in the spermatid no vacuolar material can be demonstrated by the neutral-red test. During all this time, however, the presence of Golgi material is clearly shown by the standard methods. Furthermore, when the earliest 'vacuolar' material reappears in the spermatid, it is always seen inside the acroblast, which was developed by aggregation of Golgi material and was clearly visible before the appearance of the vacuoles.

Parat believes that the so-called Golgi apparatus of the male germ cells is not a distinct cytoplasmic element, but merely a group of modified chondriosomes, to which he gives the name lepidosomes. This view is based on three lines of evidence, as follows: first, a chemical similarity as shown by the tendency of both to impregnate with silver and osmic acid and to stain with certain basic dyes, and in fresh preparations with Janus green; secondly, a morphological likeness based on the assumption that the chondriosome type is filamentous and the typical Golgi body crescentic; thirdly, observations of Parat and his associates (Gambier, '28, and Parat, '28) that in the spermatogonia or early spermatocytes of some animals all intermediate stages between the crescentic Golgi bodies and the filamentous chondriosomes can be made out.

The evidence from a similarity of staining reaction is certainly not conclusive. Janus green is the only dye that is generally admitted to have a high degree of specificity for chondriosomes. It has been generally reported by Parat's school that the Golgi bodies of the male germ cells are stainable with Janus green in fresh preparations. Other observers (notably Hirschler and Monné) maintain that this reaction occurs only when the staining has proceeded so far that the general cytoplasm, nucleus, etc., are tinted. I have found the same to be true in Gerris. Under such conditions it is doubtful whether this similarity in reaction of the chondriosomes and Golgi bodies could be held to have much significance.

In a previous section of this paper it was pointed out that most of the cases in which the Golgi bodies were formerly supposed to be crescentic have been shown to be really examples of a univesicular apparatus. In other cases the Golgi material is in the form of scattered vesicles, but in no case has the presence of crescentic bâtonettes such as those previously described by Gatenby in Helix and more recently by Parat and Painlévé in this form and in Amphibia and mammals been certainly established by the most modern methods. In view of this situation, Parat's argument from a morphological resemblance between Golgi bodies and chondriosomes is considerably weakened. In Gerris there is at no time any sort of morphological resemblance between the two, although, of course, it must be admitted that this study has not given much attention to the situation in the spermatogonia and early spermatocytes. Criticism of the third line of evidence is involved with that of the second. Parat ('28) and Gambier ('28) have described the existence of intermediate stages between the ordinary chondriosomes and the lepidosomes in the early spermatocytes of Helix and Discoglossus. This has been based on the description of the early Golgi bodies as consisting of a group of crescentic bodies surrounding the idiosomic region. In view of the fact that others (Murray, '98; Avel, '25; Monné, '28; Hirschler, '28) describe the Golgi apparatus in the same or closely related forms as consisting of a wrinkled envelope, these 'transitional stages' should certainly be more carefully studied than they have been by Parat and Gambier. In their present state these certainly do not appear very convincing, and, unless corroborated by strong evidence of anotner sort, show very little as to a possible relationship between chondriosomes and Golgi bodies in the male germ cells.

SUMMARY

The history of the cytoplasmic components in the spermatogenesis of several members of the genus Gerris has been worked out by a combination of standard technical methods and observations of teased preparations stained with the so-called vital dyes, neutral red and Janus green.

The chondriosomes are granular and grouped in a mass at one pole in the earliest spermatocytes. During the early growth period the granules become dispersed throughout the cytoplasm. The granules increase in number and elongate to form filaments, which undergo considerable increase in diameter. In Gerris remigis these filaments break up to form spheres, after reaching their maximum diameter. In Gerris marginatus the filaments persist throughout the maturation divisions.

In the cells undergoing mitosis the chondriosomes maintain a very definite topographical relationship to the centrioles.

In the spermatid the chondriosomes fuse to form a compact nebenkern of the usual insect type. This body undergoes a series of structural changes similar to those previously described for other forms and culminating in the formation of two twisted chondriosome threads extending nearly the entire length of the sperm tail.

The early spermatocyte Golgi material consists of a number of spherical or ovoid structures, the Golgi *bodies*, each of which has an osmiophilic periphery and a central non-osmiophilic material probably stainable with neutral red in fresh preparations. During the growth period the Golgi bodies increase greatly in size and change their shape to that of irregularly folded, sac-like structures.

During the prophase of the first maturation division the Golgi bodies fuse to form several much larger masses arranged in a belt around the equator of the spindle. These masses are clearly visible in fresh preparations. Their contents are stainable with neutral red. The peripheral parts of these bodies cannot be stained in fresh preparations, but can be blackened with osmic acid after appropriate fixation.

Prior to the anaphase of the first maturation division the Golgi masses break up into many fragments (dictyosomes), which are then dispersed at random throughout the cell, except in the areas of the asters and spindles, from which they are absent. The dictyosomes are apparently distributed passively to the secondary spermatocytes and spermatids. The neutral-red-staining components of the Golgi aggregates do not accompany the peripheral osmiophilic portions during the fragmentation, but remain at the equator of the first division figure, where they probably disintegrate. This suggests that the Golgi apparatus of the spermatocytes of Gerris is not essentially duplex in its morphological structure.

In the early spermatids the osmiophilic Golgi material becomes aggregated to form an irregularly folded, sac-like structure, the acroblast. During the later stages of spermiogenesis the origin and growth of the acrosome occur in close association with the acroblast. After the formation of the acrosome is completed, the acroblast is separated from contact with it, and passes down the sperm tail with the residual cytoplasm. Observations of fresh preparations indicate that the acroblast is composed of an elastic material, which is highly resistant when isolated in physiological salt solution.

The acrosome is a complex of two main masses of material, the sheath and the core, each of which shows further differentiations. The acrosome is unusually large in Gerris. It forms nearly one-half of the length of the mature spermatozoon.

As the axial filament grows out it carries a small bulb on its tip. The origin, fate, and general significance of this bulb are obscure.

In the spermatocytes of Gerris marginatus there are one or more cytoplasmic structures, that are provisionally called chromatoid bodies. These are sickle-shaped, and may be divided in one of the maturation divisions if caught in the plane of constriction.

In fresh preparations the centrioles are clearly visible during the maturation divisions.

LITERATURE CITED

AVEL, M. 1925 Sur les propriétés physiques de l'appareil de Golgi. C. R. soc. biol., T. 93.

BĚLAŘ, K. 1927 Beiträge zur Kenntnis des Mechanismus der indirekten Kernteilung. Naturwissenschaften, Bd. 15.

BOWEN, R. H. 1920 Studies on spermatogenesis. I. Biol. Bull., vol. 39.

——— 1922 a On certain features of spermatogenesis in Amphibia and insects. Am. Jour. Anat., vol. 30.

——— 1922 b Studies, etc. II. Jour. Morph., vol. 37.

——— 1922 c Ibid. III. Q.J.M.S., vol. 66.

——— 1923 The origin of secretory granules. Proc. Nat. Acad., vol. 9.

——— 1924 Studies, etc. VI. Jour. Morph. and Physiol., vol. 39.

1926 The Golgi apparatus—its structure and functional significance. Anat. Rec., vol. 32.

CHAMBERS, R. W. 1923 General cytology. Univ. of Chicago Press.

CHAMPY, C. 1913 Recherchés sur la spermatogénèse des batrachiens. Arch. d. zool. exp., T. 52.

CHICKERING, A. M. 1927 Spermatogenesis of the Belostomatidae. Jour. Morph. and Physiol., vol. 44.

GAMBIER, E. 1928 Sur la constitution de l'idiosome, etc. C. R. Soc. biol., T. 98.

Gatenby, J. B. 1917 a Cytoplasmic inclusions of the germ cells. I. Q.J.M.S., vol. 62.

——— 1917 b Ibid. II. Ibid., vol. 62.

——— 1922 Gametogenesis of Saccocirrus. Ibid., vol. 66.

——— 1925 Spermatogenesis of Peripatus. Ibid., vol. 69.

Gatenby, J. B., and Battacharya, D. R. 1925 Notes on cytoplasmic inclusions. La Cellule, T. 35.

Hibbard, H. 1922 Cytoplasmic inclusions in the egg of Echinarachnius. Jour. Morph., vol. 36.

Hirschler, J. 1918 Ueber den Golgischen Apparat enbryonaler Zellen. Arch. f. mikr. Anat., Bd. 91.

——— 1927 Studien ueber Golgi Apparat und Mitochondrien. Zeit. f. Zellf., Bd. 5.

——— 1928 a Appareil de Golgi—vacuome au cours de spermatogénèse. C. R. soc. biol., T. 37.

——— 1928 b Studien über die Plasmakomponenten. Zeit. f. Zellf., Bd. 7.

Hirschler, J., and Monné, L. 1928 Studien über die Plasmakomponenten. Zeit. f. Zellf., Bd. 7.

Hyman, O. W. 1923 Spermic dimorphism in Fasciolaria tulipa. Jour. Morph., vol. 37.

Karpowa, L. 1925 Beobachtungen über den Apparat Golgi (Nebenkern). Zeit. f. Zellf., Bd. 2.

Lewis, M. R., and Lewis, W. H. 1915 Mitochondria in tissue cultures. Am. Jour. Anat., vol. 17.

Lewis, M. R., and Robertson, W. R. B. 1916 The mitochondria and other structures observed by the tissue culture methods in male germ cells of Chorthippus. Biol. Bull., vol. 30.

Ludford, R. J., and Gatenby, J. B. 1921 Dictyokinesis in germ cells, etc. Proc. Roy. Soc., B, vol. 92.

Meves, F. 1896 Ueber die Entwickelung der männlichen Geschlechtzellen von Salamandra. Arch. f. mikr. Anat., Bd. 48.

——— 1907 Die Spermatocytenteilungen bei der Honigbiene. Arch. f. mikr. Anat., Bd. 70.

Monné, L. 1927 Observations sur les spermatocytes des Mollusques. C. R. soc. biol., T. 97.

Montgomery, T. H. 1911 The spermatogenesis of an Hemipteron, Euschistus. Jour. Morph., vol. 22.

Morelle, J. 1925 Les constituants du cytoplasme dans le pancreas et leur intervention dans le phénomène de secretion. La Cellule, T. 37.

Morgan, L. O. 1925 On the cytoplasmic inclusions in the male germ cells, etc. Anat. Rec., vol. 30.

Murray, J. A. 1898 Contributions to the knowledge of the nebenkern, etc. Zool. Jahrb., Bd. 11.

Pantel, J., and de Sinety, R. 1906 Les cellules de le lignée mâle chez le Notonecta. La Cellule, T. 23.

Parat, M. 1926 Sur la constitution de l'appareil de Golgi et de l'idiosome. C. R. acad. sci., T. 182.

PARAT, M. 1928 Contribution à l'étude morphologique et physiologique du cytoplasme. Arch. d'anat. mic., T. 24.

PARAT, M., ET GAMBIER, E. 1926 L'appareil de Golgi des cellules génitales mâles. C. R. soc. biol., T. 94.

PARAT, M., AND PAINLÉVÉ, J. 1926 Ibid. C. R. soc. biol., T. 94.

PAYNE, F. 1927 Some cytoplasmic structures in the male germ cells of Gelastocoris. Jour. Morph. and Physiol., vol. 43.

SHAFFER, J. 1920 The germ cells of Cicada. Biol. Bull., vol. 38.

SJÖVALL, E. 1906 Ein Versuch, das Binnennetz von Golgi-Kopsch bei der Spermato- und Ovogenese zu homologisieren. Anat. Anz., Bd. 28.

SOKOLOV, I. 1926 Untersuchungen über die Spermatogenese bei den Arachniden. Zeit. f. Zellf., Bd. 3.

SOKOLSKA, J. 1924 Über Ausbleiben der Teilung. Arch. mikr. Anat., Bd. 103.

SPEK, J. 1918 Oberflächenspannungsdifferenzen als Ursache der Zellteilung. Arch. f. Entw., Bd. 44.

TUZET, O. 1927 Sur la double spermatogénèse du Mollusque prosobranche. Arch. d. zool. exp., T. 67.

——— 1928 Observation, sur le vivant, des composants cytoplasmiques. C. R. soc. biol., T. 97.

VOINOV, D. 1925 Les elements sexuelles de Gryllotalpa vulgaris. Arch. d. zool. exp., T. 63.

——— 1927 Le vacuome et l'appareil de Golgi dans les cellules génitales. Ibid., T. 67.

WILKE, G. 1913 Chromatinreifung und Mitochondrienkörper in der Spermatogenese. Arch. f. Zellf., Bd. 10.

WILSON, E. B. 1916 The distribution of the chondriosomes to the spermatozoa. Proc. Nat. Acad., vol. 2.

——— 1925 Protoplasmic systems and genetic continuity. Amer. Nat., vol. 59.

PLATES

EXPLANATION OF PLATES

All the figures were outlined as far as possible with the camera lucida and the details filled in freehand. Plates 1 to 5 are drawn at a total magnification of 2600 diameters. Plate 6 is drawn at a magnification of 2300 diameters.

ABBREVIATIONS

AB, acroblast	*CE*, centriole
AC, acrosome	*N*, nucleus
AF, axial filament	*PA*, pro-acrosomic material
C, chromatoid body	*SA*, sheath of acrosome
CA, core of acrosome	

PLATE 1

EXPLANATION OF FIGURES

All the figures are from Kull preparations of the testes of Gerris remigis.

1 Two early spermatocytes, showing granular chondriosomes grouped at one pole of the cell. The cells are connected by a 'Spindelrest.'

2 Early spermatocytes, showing the beginning of the dispersal of the chondriosomes throughout the cytoplasm.

3 Spermatocyte in the early growth period. The chondriosomes have elongated to form slender filaments scattered throughout the general cytoplasm.

4 Spermatocyte slightly later than figure 3, showing increase in size and number of chondriosomes.

5 Spermatocyte near the completion of the growth period. The chondriosomes have increased greatly in diameter, attaining their maximum size as filaments. The spheres outlined with heavy lines are Golgi bodies, the osmiophilic peripheries of which were stained with acid fuchsin.

6 A stage slightly later than that of figure 5. Some of the chondriosomes are being broken up into spheres. Two Golgi bodies are visible, though unstained.

7 Polar view of late prophase of the first maturation division. This cell illustrates the orientation of the chondriosomes radial to the centrioles.

8 A lateral view of the metaphase of the first division, showing especially the orientation of the chondriosomes. The granules in the equatorial region are probably fragments of Golgi bodies.

9 A lateral view of the early anaphase of the first division.

10 Late anaphase of the first maturation division.

11 Lateral view of secondary spermatocyte, showing early stage in separation of the centrioles as the first step toward reorientation for the second division. Note that the orientation of the chondriosomes is slightly altered in response to this movement.

12 Secondary spermatocyte, with separation of centrioles nearly completed. Reorientation of chondriosomes practically finished, but the chromosome plate has not yet rotated. In this and the following figure the astral rays are exaggerated to show the extent of the astral areas.

13 Slightly oblique view of a stage a little later than figure 12.

CYTOPLASMIC PHENOMENA IN GERRIS PLATE 1
ARTHUR W. POLLISTER

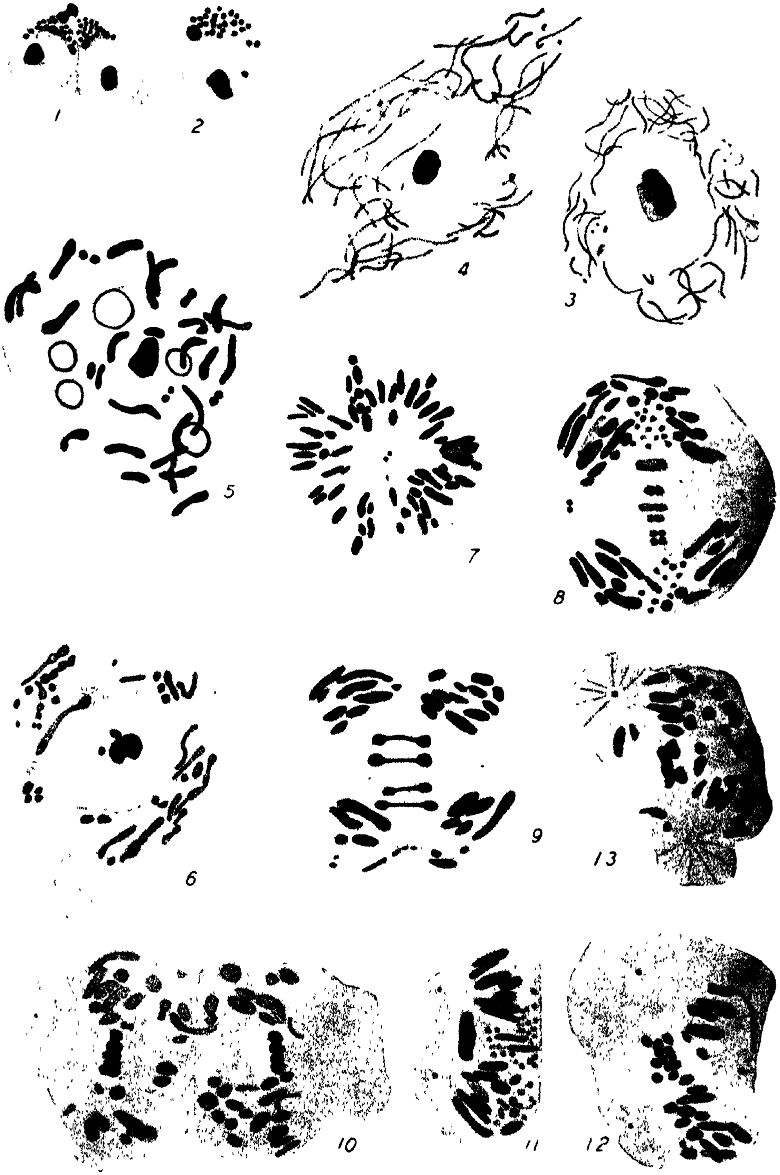


PLATE 2

EXPLANATION OF FIGURES

Figures 14 to 21, 31, and 36 are from Kull preparations of Gerris remigis Figures 22 to 25 are from Benda preparations of Gerris marginatus. Figures 26 and 33 are from Flemming-hematoxylin preparations of Gerris marginatus. Figure 32 is from a Champy-hematoxylin preparation of Gerris marginatus. Figure 35 is from a Kull preparation of Gerris marginatus. Figure 34 is from a Weigl preparation of Gerris remigis.

14 Secondary spermatocyte, viewed from the pole of the first division figure. This should be compared with figure 17 to emphasize the fact that the reorientation of the chondriosomes is completed before rotation of the chromosome plate.

15 and 16 Lateral and polar views, respectively, of the second maturation division metaphase. Note especially the asymmetrical arrangement of the chondriosomes. Compare with figures 7 and 8.

17 and 18 Views of metaphase and anaphase, respectively, of the second maturation division, viewed from the pole of the first division figure.

19 Polar view of anaphase of the second maturation division.

20 Lateral view of two sister spermatids, showing intermediate stage in the peripheral movement of the centrioles (*CE*) and the rotation of the chromosome plates (*N*) (telokinetic movements). Note accompanying movement of the chondriosomes.

21 Two sister spermatids viewed from the side of the second division figure. Note that the centrioles (not drawn in the figure), chromosome plates, and chondriosomes have the same topographical relationships as at the early telophase of the second division (fig. 19). The chondriosomes show a differentiation into chromophilic and chromophobic substances.

22 Spermatid at stage slightly later than figure 21. Note differentiation of all the chondriosomes into two materials.

23 and 24 Two different views of early spermatids, showing clumping of chondriosomes as initial stage in nebenkern formation.

25 and 26 Two different views of the hollow ring structure formed by close aggregation of the chondriosomes.

27 to 30 Probable successive stages in establishment of the 'spireme' type of nebenkern pattern.

31 Spermatid, showing the nebenkern shortly after the appearance of the central substance.

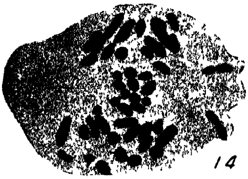
32 Intermediate stage in development of central substance and disappearance of chromophilic platework. Note indication of bipartite division of nebenkern, and also axial filament with end bulb.

33 Spermatid of Gerris marginatus, showing uniform elongation of the two nebenkern halves.

34 Nebenkern of Gerris remigis, showing spinning out of the posterior ends of the two halves to form beaded ribbons.

35 Portion of tail of late spermatid, showing beaded-ribbon stage. Note that the ribbons are twisted about each other.

36 Portion of tail of late spermatid after formation of twisted chondriosomal ribbons with nodal swellings.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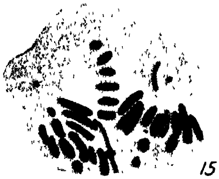
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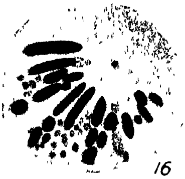
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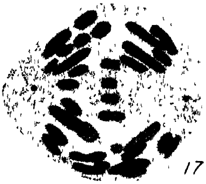
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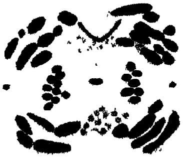
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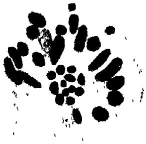
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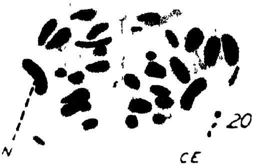

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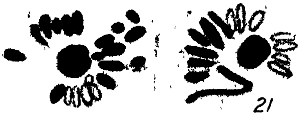
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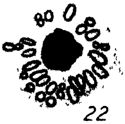
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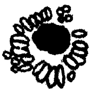
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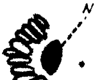

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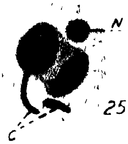

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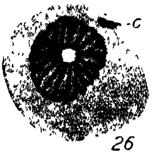

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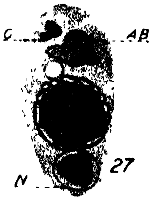

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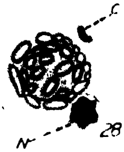

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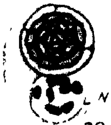

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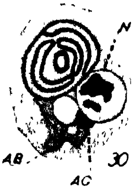

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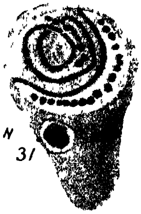

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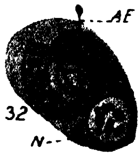

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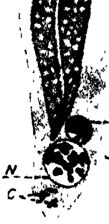

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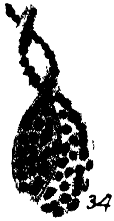
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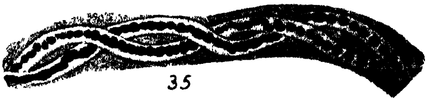
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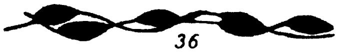
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PLATE 3

EXPLANATION OF FIGURES

Figures 37 and 38 are from Kull preparations of Gerris marginatus. All the other figures are from Weigl preparations of Gerris remigis.

37 and 38 Early spermatocytes, showing scattered spherical and ovoid Golgi bodies.

39 to 41 Successive stages in growth of primary spermatocytes, showing increase in number and size of Golgi bodies. Note differentiation of each body into osmiophilic periphery and non-osmiophilic core.

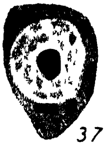
42, 43, and 45 Later stages of growth of spermatocytes, accompanied by increasing size and change of shape of Golgi bodies.

44 Single Golgi bodies, illustrating possible obliteration of early non osmiophilic portion.

46 and 47 Lateral and polar views, respectively, of late prophase of the first maturation division. The Golgi bodies have fused together to form several irregular masses in the equatorial region of the cell.

48 Lateral view of the metaphase of the first maturation division, showing an intermediate stage in the fragmentation of the Golgi masses and the dispersal of the fragments (dictyosomes).

49 The same view as figure 48, but after completion of the fragmentation of the Golgi masses.



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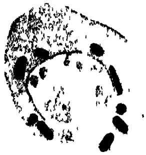

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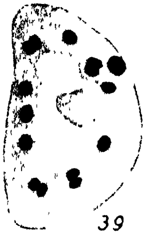

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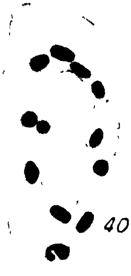

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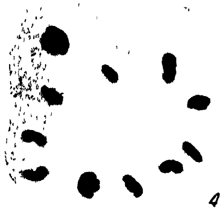

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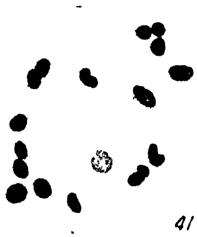

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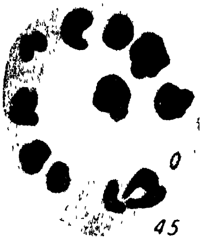

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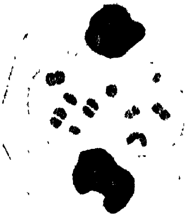

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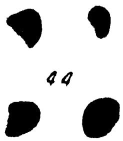

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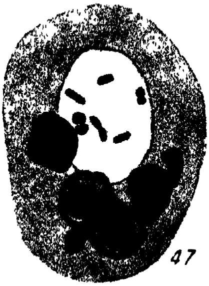

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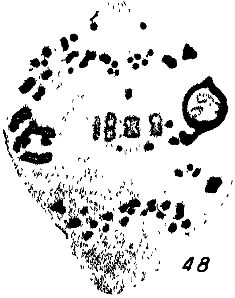

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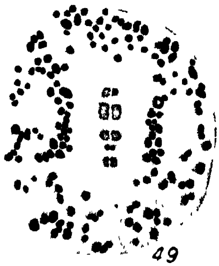

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PLATE 4

EXPLANATION OF FIGURES

All figures are from Weigl preparations of *Gerris remigis*.

50 Late anaphase of the first division. Note random distribution of dictyosomes.

51 Metaphase of second maturation division.

52 Early sister spermatids, before aggregation of dictyosomes.

53 to 56 Early spermatids, showing successive stages in aggregation and fusion of dictyosomes to form the aeroblast (*AB*, fig. 56).

57 Late stage in formation of aeroblast. Note pro-aerosomic material connected to masses formed by aggregation of dictyosomes.

58 Completed aeroblast (*AB*) with pro-aerosomic material inside it.

59 Early spermatid, showing an early stage in growth of aerosome (*AC*). In this figure (and in figs. 60 and 63) the lighter rim around the aerosome is probably an artifact due to shrinkage.

60 and 61 Stages in aerosome formation slightly later than figure 59. Figure 60 illustrates the contemporary pattern of the nebenkern.

62 Stage slightly later than figure 61, showing the first demonstrable heterogeneity in the aerosome. The aeroblast contains pro-aerosomic material.

63 Later stage in growth of aerosome (*AC*). Note smaller pro-aerosomic sphere inside the aeroblast.

64 Aerosome (*AC*) at its maximum size. Note aeroblast (*AB*) still attached to one side of it.

65 Very late spermatid, showing aeroblast fragments (*AB*) separated from completed aerosome (core, *CA*, plus sheath, *SA*) and passing down the sperm tail.

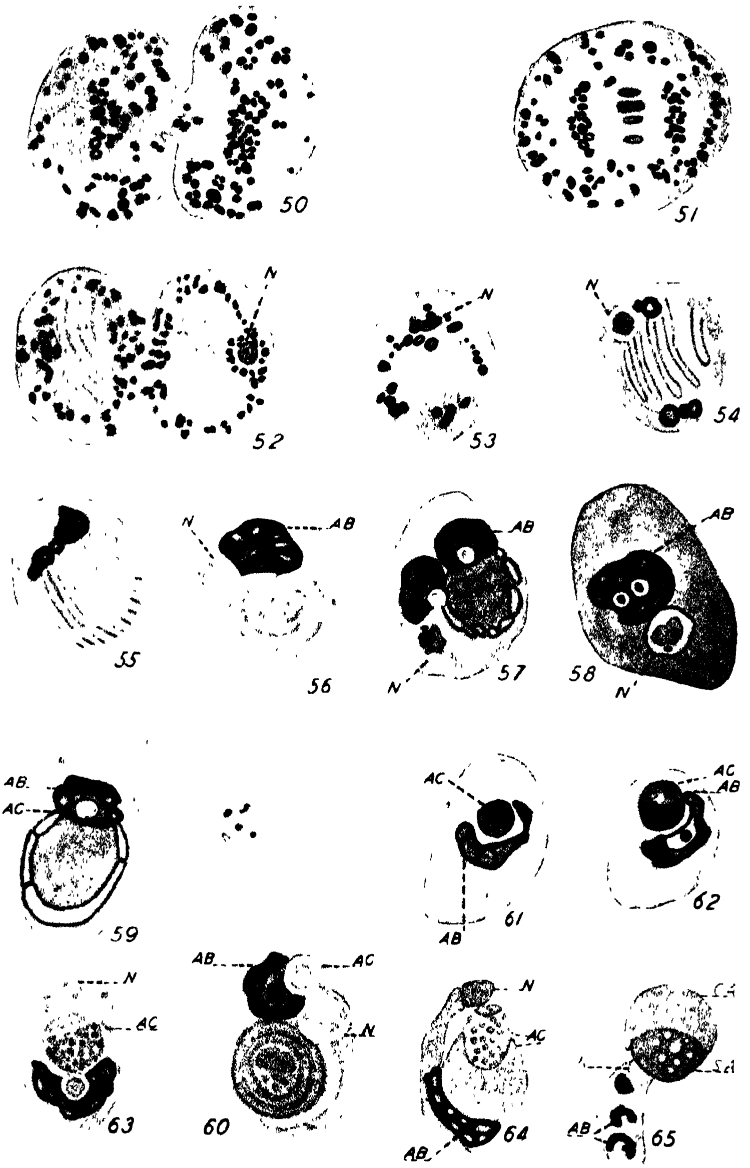
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PLATE 5

EXPLANATION OF FIGURES

Figures 66 to 71 are from Benda preparations of Gerris marginatus. Figures 72 and 73 are from Weigl preparations of G. remigis. Figures 75 to 78 are from Bouin-hematoxylin preparations of G. remigis. Figures 74, 79, and 80 are from Kull preparations of G. remigis.

66 Spermatocyte in middle growth period, showing sickle-shaped chromatoid body (*C*).

67 Metaphase of second maturation division, with chromatoid body in such a position that it will pass wholly into one spermatid at cell division.

68 Telophase of second division. The chromatoid body (*C*) is entirely in one spermatid a condition foreshadowed in figure 67.

69 Telophase of second maturation division, in which one chromatoid body has gone entirely into one spermatid, while the other is being divided by the cell constriction.

70 Spermatid showing two masses of chromatoid substance (*C*). Also note end bulb of axial filament.

71 End of tail of spermatid with nebenkern at beaded-ribbon stage. Note end bulb of axial filament (*AF*).

72 and 73 Two views of acrosome (core, *CA*, plus sheath, *SA*) just prior to elongation.

74 Head of very late spermatid, in which the core of the acrosome (*CA*) has straightened out, while the substance that later becomes the sheath is in the form of a sphere (*SA*) attached to one side of the core.

75 Stage slightly later than figure 74. The sheath (*SA*) has drawn out along one side of the core (*CA*).

76 and 77 Two views of a stage approximately like that of figure 75, showing differentiation within the core of the acrosome (*AC*). The sheath was not distinguishable, as it was stained like the core.

78 Early stage in elongation of the acrosome (*AC*).

79 and 80 Two successive stages in elongation of nucleus (*N*) and adjacent part of acrosome (*AC*).

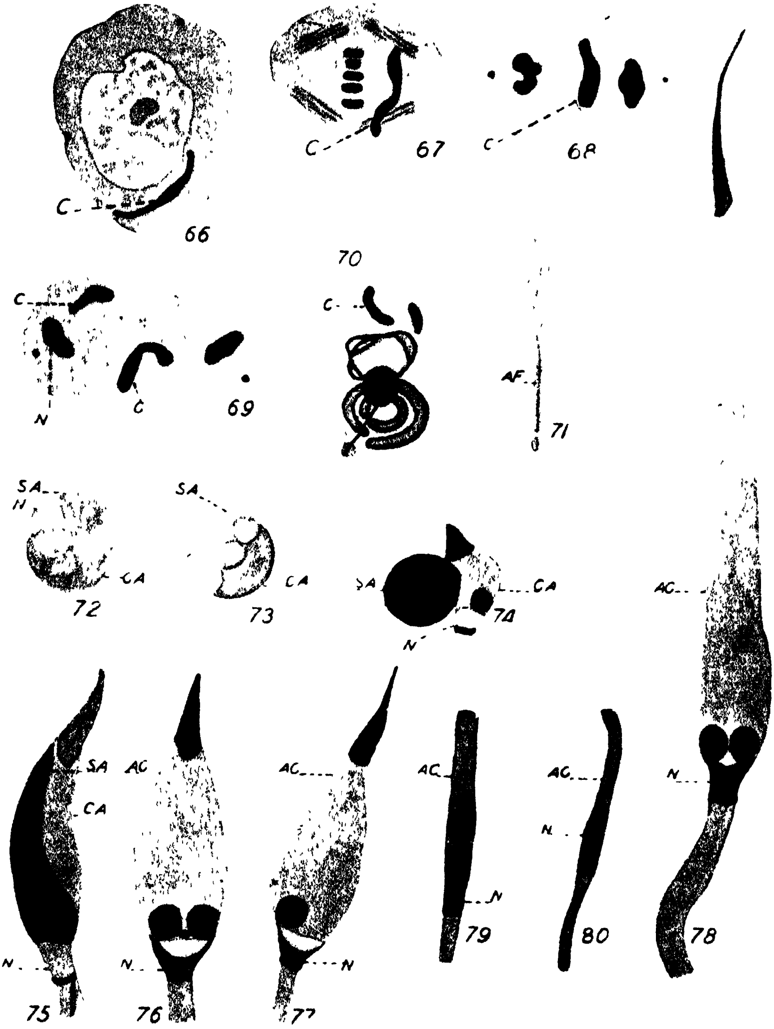
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PLATE 6

EXPLANATION OF FIGURES

All the figures are drawn from freshly teased preparations of Gerris marginatus that had been lightly stained with neutral red and Janus green. In all except figures 81 and 83 the colored substance is either acrosomic or pro-acrosomic material.

81 Early spermatocyte, showing beginning of dispersal of granular mitochondria throughout the cytoplasm.

82 Spermatocyte slightly later than figure 81. The scattering of the chondriosomes has been completed.

83 Late prophase of the first maturation division, showing the filamentous chondriosomes and their orientation with respect to the clear polar centrosome regions. Note the polar filaments and, around the equator, the Golgi masses with contents stainable with neutral red.

84 Telophase of second maturation division, showing telokinetic movements of centrioles, chromosome plates, and chondriosome mass. The spindle is somewhat schematized.

85 Early spermatid with nebenkern at 'spireme' stage. Note also the acroblast (*AB*) with early acrosome, the latter stained with neutral red.

86 and 87 Successive later stages in the formation of pro-acrosomic and acrosomic materials. Also note the end bulb of the axial filament (*AF*).

88 Spermatid with divided nebenkern, containing central substance as light droplets.

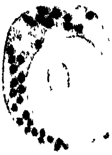
89 Spermatid showing nebenkern halves in early stage of elongation. Note increasing size of acrosome (*AC*) and many pro-acrosomic spheres inside the acroblast (*AB*).

90 Acroblast isolated from cell by crushing, as described in text. The acrosome is attached at one side and pro-acrosomic droplets are inside the acroblast.

91 Late stage in formation of acrosome (*AC*). Note its differentiation into two main parts.

92 Spermatid at stage when the growth of the acrosome has reached its maximum. The acrosome is slightly distorted because of rounding-up of cell. Note twisted nebenkern filaments, which were stained by Janus green.

93 Late spermatid, in which the acroblast fragments (*AB*) have separated from the acrosome, whose core (*CA*) and sheath (*SA*) are clearly distinguishable because of a marked difference in refractive index.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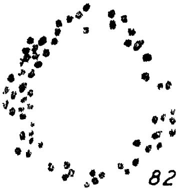
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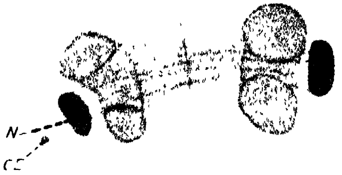

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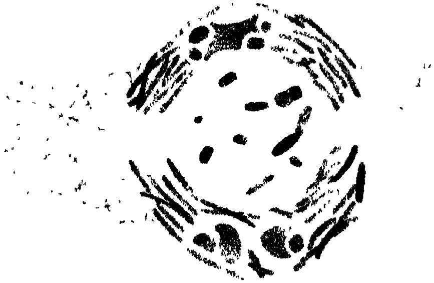
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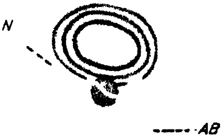

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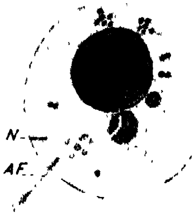

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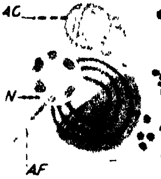

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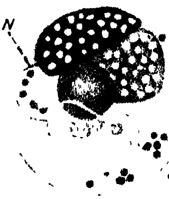

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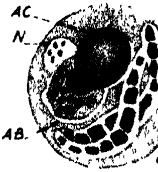

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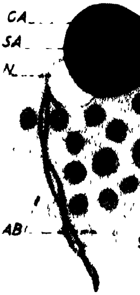

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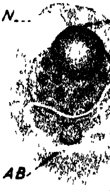

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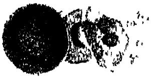
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CYTOLOGICAL STUDIES ON THE SPINNING GLANDS OF THE LARVA OF GALLERIA MELLONELLA: RESPECTIVE RÔLES PLAYED BY THE NUCLEUS, THE GOLGI APPARATUS, AND THE MITOCHONDRIA DURING SECRETION

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Zoological Laboratory, University of Wisconsin

THREE PLATES (TWENTY-EIGHT FIGURES)

AUTHOR'S ABSTRACT

Spinning in Galleria begins shortly after hatching and continues throughout larval life. The gland cells secrete continuously, irrespective of the act of spinning

The nucleus plays a direct and an important rôle in silk secretion by the migration of nucleoli into the cytoplasm, where they enlarge and synthesize a fatty material in the center, the fatty material is transformed into a non-soluble basophilic substance, which then changes into the secretory product in its final form. The processes of converting the fat into non-soluble substance and of converting the latter into the secretory product progress inwardly from the periphery of each secretory body. The secretory bodies or masses of secretory material break up into smaller and smaller masses and eventually into a fine dispersed state before their entrance into the lumen of the gland

The mitochondria are granular in the cells of the conductive portion and filamentous in those of the reservoir and secretory portions of the gland. In the secretory portion they are orientated with the long axis toward the gland lumen. Their rôle in silk secretion is negligible or at most a minor one

The Golgi apparatus is in the form of discrete ring- and half ring-shaped bodies and remains so during all stages of secretion. If it plays any rôle in silk secretion, the fact has not been detected by the author.

INTRODUCTION

In previous studies on the spinning glands of the larva of the caddis fly, Platyphylax designatus Walker (Beams and Wu, '29), it was found that, during physiological activity, the nucleus participates directly in secretion by the migration of nucleoli into the cytoplasm, where they undergo chemical changes and then dissolution. The Golgi apparatus in the form of discrete bodies, although becoming slightly more dispersed in the advanced stages of forced physiological activity, does not display any other change, such as hypertrophy, definite polarity, or direct connection with the secretory bodies. The findings of the present studies on the spinning glands of the larva of the wax moth or bee moth, Galleria mellonella, confirm, in a general way, the results of our studies on the spinning glands of the caddis worm, namely: the nucleoli migrate from the nucleus out into the cytoplasm, where they undergo a series of elaborate chemical changes to form the secretory substance which is subsequently poured into the lumen of the gland; the Golgi bodies, here again, do not show any apparent hypertrophy, definite polarity, or direct connection with the secretory substance during secretion; and the mitochondria, which were not included in our studies on the caddis worm, offer to show some connection with the secretary phenomenon, although only in a slight degree.

The direct rôle played by the nucleus in the secretion of spinning glands of insects has repeatedly been suggested or definitely demonstrated by various authors, chief among whom are Gilson ('90), Marshall and Vorhies ('06) Maziarski ('11), Nakahara ('17), Kinney ('26), Noel and Paillot ('27, '28), and Dehorne and Hosselet ('28); the last, particularly, have demonstrated, by means of vital staining, the migration of nucleoli into the cytoplasm, where they undergo a series of elaborate chemical as well as morphological changes finally to form the secretory substance. The rôle of mitochondria in the secretion of spinning glands has been studied by Kinney ('26), and more thoroughly by Noel and Paillot ('28), and by Dehorne and Hosselet ('28). As regards the Golgi bodies, Beams and Wu ('29) were the first to describe and to study their behavior in the spinning glands.

The author wishes to express his indebtedness to Prof. M. F. Guyer, under whose direction this work was undertaken, to Prof. W. S. Marshall, who has given much good counsel and advice, and, finally, to Mr. H. W. Beams, for much help and criticism.

MATERIAL AND METHODS

The glands of the larvae of Galleria mellonella were used in these studies, because these larvae are of suitable size, easily reared, readily obtained in large numbers, and particularly because they are very good spinners. Spinning begins shortly after hatching and continues throughout the larval life except during the short intervals of ecdyses. The larvae were raised in battery jars and glass utensils of like kind in which pieces of old bee's combs had been placed. The entire life cycle of Galleria mellonella is passed in six to seven weeks. The various stages of the life cycle may be distributed in the following manner, according to Metalnikov ('08): eight to ten days during the egg stage, twenty-one to twenty-five days during the larval stage, and ten to twenty-five days during the chrysalid stage. By varying the abundance of food and temperature, however, one can shorten or prolong the larval life of this insect.

It is generally known that the physiology of secretion runs in cycles and that in each cycle there is a series of events, one leading to the other. To study successfully this series of events or the successive phases of each secretory cycle, one of the following two conditions should be present. In the first case, speeding up of the secretory activity, so that the various phases of the cycle will be run in rapid succession, will favor the chance of having these phases 'caught' in action. Secondly, the same end may be accomplished by prolonging the duration of the successive phases of the secretory cycle. With these things in mind, I first tried what I termed 'forced activity' experiments. Well-fed larvae of medium size were put in finger-bowls with half an inch of clean, white sand on the bottom. The larvae would start spinning immediately in an attempt to make cocoons. The cocoons or rather masses of silk and sand grains were removed at regular intervals of six to ten hours, so that the larvae were constantly kept spinning. They were not fed. They were then taken out and killed at intervals of 6, 12, 24, 36, 48, 72, 100, and 200 hours; by the end of the last-mentioned time, most of the larger larvae had gone into pupation.

In an attempt to secure glands in a true resting state for comparison with the active glands, a number of larvae were put in a cold-room which had a temperature of 5° to 7°C. At this low temperature, the larvae become motionless in a short time and lost all signs of activity. By the end of seven days, they were taken out and killed. Upon dissection, it was found that the glands were about two to three times as large as those of the normally active larvae, and that this was especially true with the middle portion of the gland. On studying the prepared sections of the secretory portion of the gland, to my great surprise I found that the cells were literally filled with secretory inclusions of all sizes. After considerable scrutiny, I finally came to the conclusion that the silk glands of the waxmoth larva secrete continuously, irrespective of spinning. What happened in this cooling experiment was simply this: the low temperature of the cold-room was just cold enough to paralyze and prevent the larvae from spinning, but not quite cold enough to stop the secretory activity of the gland cells as well. The secretion, which was continuously elaborated by the gland cells, became accumulated in the lumen of the gland. As this process went on, the passing of the secretory substance from the cell into the lumen became increasingly difficult and slow. As a result, the secretory bodies became accumulated in the gland cells as well and tended to remain where they had been elaborated. With this information at hand, I then began a series of similar experiments with larvae taken out of the cold-room and killed at intervals of twelve hours, one day, three days, and seven days.

That the gland cells secrete continuously and independently of the act of spinning further suggested another line of experiments, namely, that of preventing the larvae from spinning at room temperatures. This was accomplished by putting, just behind the head, a ligature which was barely tight enough to prevent spinning. This series of experiments was not, however, completely carried out, and only the glands of larvae ligated for twenty-four hours were thoroughly studied. Such a ligation method was not original with the author, but had long ago been employed by Gilson ('90) in his studies of the spinning glands of the silkworm. Recently, Chorine and Korvine-Kroukovsky ('29), working on the immunization of fragments of the body of the wax-moth larva, found that larvae decapitated and ligated at the cut end lived for as long as eighty-three days. It thus seems reasonable to assume that the secretory activity of the gland cells of larvae ligated for only a day or so cannot be far from normal.

The glands were removed in the following manner: the larva was first decapitated, its posterior end was firmly grasped between a pair of strong forceps; a second pair of curved forceps was applied just in front of the first pair and, with a quick forward movement of the second pair, the entire internal contents of the larva were gently pressed out of the body into normal saline. They were then either thrown into the proper fixative along with the other internal contents or, in the case of expensive fixing fluids, such as those containing osmic acid, were dissected free from the fat-bodies and the intestine, and the proper portions of the glands were then thrown into the fixative. Glands dissected out in this manner proved to be good for all purposes, except for the study of mitochondria, which, in this case, underwent a marked contraction and appeared, in the prepared sections, in the form of short rods or even granules instead of the usual form of long filaments.

The fixatives employed were the following. For general histology and cytology, Gilson's, Bouin's, strong Flemming's with and without acetic acid, Mann's, and Champy's were used. For mitochondria, Schridde's and Regaud's were largely used; Mann's and Champy's were also good for fixing mitochondria, particularly the latter fixative; but, because of the necessity of dissecting the glands in these cases, the mitochondria were generally in the contracted state, due, evidently, to the mechanical injury to the gland in dissection. For Golgi bodies, Ludford's modification of the Mann-Kopsch method, which had proved to be the best in our study of the caddis worm, was exclusively used. I had, however, to modify the time schedules as given by Ludford ('26). The tissue was fixed in Mann's for twelve to eighteen hours at room temperature, washed for two hours in a large amount of distilled water, and then put into 2 per cent osmic acid at 35°C. for four days and up. From the third or fourth day on, small pieces of the tissue were examined in glycerin from time to time to determine the degree of impregnation, as suggested by Nassonov. For staining, iron haematoxylin was chiefly used both for general cytology and for the study of mitochondria. Other stains which proved to be useful were safranin, toluidin blue, and a number of the common plasma dyes.

After fixation and washing as the case demanded, the tissue was dehydrated through grades of alcohols at ten to thirty minutes each. Clearing was done in chloroform. The tissue was then emptied into a cone, made by twice folding a piece of lens paper of 2 by 2 inches, which was dipped immediately into a dish of chloroform saturated with paraffin. After five or ten minutes, the cone was transferred into melted paraffin of 52°C. for five to ten minutes. In embedding, the cone was removed from the bath, unfolded, and the side to which the pieces of tissue were attached was lowered into the embedding dish which contained paraffin with a melting-point of 58°C. The high temperature of the embedding paraffin loosened the tissue from the lens paper. The pieces of tissue were quickly arranged with a warm needle, and the dish was then immersed in cold water. This method of handling tissues in lens-paper cones through the infiltrating and embedding processes, proved so indispensable in the case of the Golgi preparations, may be useful to those who handle very small and brittle objects. For general cytology and for Golgi preparations, the sections were cut at 2 to 4 μ, while for the study of mitochondria they were cut at 1 to 3 μ.

The cytoplasm of the secretory portion of the gland is so basophilic that it was necessary, prior to staining, to bleach the sections lightly in order to get a satisfactory nuclear differentiation. Sections were passed down to water and transferred to a 0.5 per cent solution of potassium permanganate for a minute or less, rinsed thoroughly in water, and immersed in a 5 per cent oxalic-acid solution until they became colorless. They were then washed thoroughly in running water and put into the stains.

A point concerning the preservation of fat may be advantageously mentioned here. It was found that xylol had a tendency to remove fat, especially when the osmication process had been short. When complete preservation was demanded, chloroform, instead of xylol, was used in removing the paraffin from the sections as well as in preparing for mounting.

OBSERVATIONS AND CONSIDERATIONS

General morphology and histology

The spinning glands of the larva of Galleria mellonella are two in number, lying along the lateroventral sides of the intestine and united at their anterior ends just behind the hypopharynx. Each gland is divisible into three regions: an anterior fine, conductive portion; a middle much swollen, reservoir portion; and a posterior secretory portion which is intermediate in size between the first two. The middle portion characteristically doubles upon itself twice, while the anterior and the posterior portions are wavy in their course, more so in the latter than in the former (figs. 1 and 2). In the full-grown larva the anterior portion is about 8 to 9 mm. in length; the middle portion, about 16 to 18 mm., and the posterior portion, about 10 to 12 mm. The glands appear as a pair of glassy, cylindrical bodies, richly supplied with tracheae both in the middle portion and in the posterior portion (figs. 5, 6, and 7). The latter portion is more or less embedded among the fat-bodies.

Each gland is formed by two longitudinal rows of cells arranged around a central lumen (fig 8.) In cross-section the wall between the cells is quite distinct near the surface of the gland, but becomes more and more indistinguishable toward the lumen. The outer and inner cell membranes of the gland, termed, respectively, 'tunica propria' and 'tunica intima' ever since the time of Helm ('76), have been exhaustively described by Gilson ('90) in the silkworm. The propria, a thin, firm membrane which is very resistant to the action of fixing fluids, is of the same structure throughout the various parts of the gland. The structure of the intima, however, differs markedly in the anterior portion and in the middle and posterior portions of the gland. In the anterior portion, it is represented by a thick chitinous layer with faint radial striations (fig. 4). Encircling this layer is a subcuticular layer or zone of radially arranged trabeculae, which was taken by Gilson to be part of the cell proper. The chitinous layer, on the other hand, has been thought by most of the previous investigators (Blanc, '89; Gilson, '90; Tanaka, '11) to be secreted by the cell. In both the middle and the posterior portions of the gland the intima is essentially a thin plasma membrane with heavily stained, spirocircular filaments, which are undoubtedly supporting structures (figs. 9, 10, and 28). Gilson ('90) described and figured, between the filaments, fibrillae which anastomose both with the heavily stained filaments as well as among themselves to form a sort of network. While I have diagramed such an appearance (fig. 10), I have reasons to believe that they are more probably a coagulation phenomenon rather than actual supporting structures of any sort. First, even in my well-stained preparations they are always faint and indistinct. Secondly, in most of the glands kept in the cold-room for three days or longer this network fails to appear altogether. Here, in the living condition, the lumen as well as the inner border of the gland cells is probably filled with the secretory substance. Due to the subsequent chemical treatment, the secretory substance undergoes a marked contraction. Hence, in the prepared sections, clear spaces appear between the columns of secretory substance and the adjacent filaments (fig. 28).

The cells, while principally hexagonal in form, vary considerably both in shape and in size in the different parts of the gland. They are small and somewhat longer than they are wide in the conductive portion, very large and flat in the reservoir portion, and less large, but thicker in the secretory portion. The cytoplasm is in all cases finely granular.

In the posterior portion it is very basophilic and gives a striated appearance which may even take on the form of fibrillae in the poorly fixed preparations.

Each cell contains a nucleus which is typically unbranched in the conductive portion and highly branched in both the reservoir and secretory portions of the gland. In each nucleus of the conductive portion there is, besides the evenly distributed chromatin granules, a large, round, heavily stained body, which is visible in the living cell and which probably corresponds to the 'nucleoloid body' of Kinney ('26) (fig. 4). In the nuclei of the other two regions of the gland two kinds of bodies are distinguishable, the chromatin granules and the nucleoli. The former are smaller and more numerous, while the latter are larger, somewhat irregular in form, size, and also in distribution, and tend to be more filamentous toward the anterior end and rounder toward the posterior end of the gland (figs. 11, 15, 18). They are, nevertheless, readily identified and are always strongly basophilic.

Mitochondria are present in all the regions of the gland. In both the middle and posterior portions they are generally orientated with the long axis toward the lumen. This orientation is not strictly followed in the middle portion, especially where the gland wall is very thin—a condition evidently brought about by the mechanical distention of the gland wall by the silk substance within the lumen. In the conductive portion numerous fine granules are present in my mitochondrial preparations. These granules are taken to be the mitochondrial elements, although they seem to be much smaller in size and more numerous than as figured by Kinney ('26).

As regards the Golgi bodies, I was successful in getting them only in the cells of the posterior portion of the gland. I was unable to impregnate anything in the conductive portion. In the reservoir portion, particularly in the anterior end of the reservoir portion, after prolonged impregnation (eight to ten days) with 2 per cent osmic acid, mitochondria, instead of Golgi bodies, were impregnated (fig. 23).

No attempt was made to analyze exhaustively the silk substance in the lumen. Briefly, the author's observations are as follows. The lumen of the secretory portion of the gland contains a thin, viscous fluid which, in sections, is lightly stained by the acid dyes and rarely by the basic ones. Toward the anterior end of this portion, there appears, in the center of this lightly stained substance, a small column of strongly basophilic substance which is in direct connection with the central-core substance of the middle portion of the gland to be described (fig. 6). Throughout the middle portion there are in the lumen two different substances according to their staining properties—the central-core portion, which is highly viscous in the living state and which is strongly basophilic, and the peripheral layer which fills the rest of the lumen and which is of the same viscosity and staining properties as the substance in the lumen of the secretory portion. Further, in studying the preparations following the cold-room treatment, I found in the cells of the middle portion the same secretory inclusions as found in the secretory portion (fig. 27). This means that the cells of the middle portion probably secrete the same chemical substances as do the cells of the secretory portion. If such an assumption be correct, then the peripheral-layer substance in the lumen of the middle portion is probably of the same chemical nature as the substance found in the lumen of the secretory portion, and both represent the condition of recently secreted stuff, which gradually transforms into the silk substance of the central core. Although numerous fat-globules are present in the cells of the anterior and posterior ends of the reservoir portion of the gland (fig. 5), no fatty substance of any kind, as tested by the osmic-acid treatment, was found in the peripheral-layer substance. The central-core substance gradually decreases in diameter and continues into the conductive portion, where it forms the silk thread. Miss Kinney ('26) reviewed the literature very thoroughly in this connection, therefore no repetition will be undertaken here.

Cytological observations

Since the sole function of the posterior portion of the gland is to secrete, any profitable cytological studies must be necessarily carried on here. Hence, the descriptions which follow are strictly applicable only to this portion of the gland.

Normally active gland. I have already mentioned the fact that the gland cells secrete continuously throughout the larval life of this insect. Under such a circumstance, it is clear that no true resting stage of the gland cell can be obtained under reasonably normal conditions. The normally active gland that I am going to describe merely represents that condition most commonly met with among glands of well-fed larvae which have not been subjected to any of the experimental conditions already described. Such a gland is typically free from secretory inclusions of any kind (fig. 11). Variable numbers of nucleoli may be present in the portions of the nucleus. Few or none of the migrated nucleoli are found in the cytoplasm. The Golgi bodies, in the form of rings and half-rings, are scattered out in the cytoplasm (fig. 19). The mitochondria are filamentous and are orientated with the long axis toward the lumen of the gland (fig. 22). It is true that secretory inclusions or other signs of cell activity may be present in some of the glands of the well-fed larvae (fig. 17), but these cases are by far very rare.

Glands in forced activity. These are the glands from larvae kept spinning constantly at room temperatures. Very little change can be observed in glands after six hours of forced activity. In glands after twelve hours of forced activity a narrow layer or zone of lightly stained, fine granular secretory substance may be seen on the inner border of a few of the cells. Such zones become wider and are present in many more cells in glands thus active for twenty-four hours. In glands of the thirty-six-hour period as many as 30 per cent of the gland cells may contain such zones which may be a third, one-half, or even two-thirds as wide as the cell itself. This form of activity probably reaches its height at the thirty-six-hour period, but may be present now and then in any of the subsequent periods up to 200 hours. At the periphery of the zone of granular secretion in certain cells little indentations are frequently seen to merge into the normal cytoplasm of the cell. Again, small patches of fine granular secretion are often seen in the central region of the cell and seem to be migrating toward this zone of secretion under discussion. From all these facts, it appears as if this finely granular secretion has been stored up in the cytoplasm of the cell and, at a certain period during the stressed activity, it moves toward the lumen side of the cell to form a solid layer which is subsequently poured into the lumen, like many thousand soldiers scattered over the countryside and, at the first bugle call, marching in battalions to the front. During this phase of activity, the nucleus does not manifest any change. Frequently, in cases where the layer of secretion is very wide, portions of the nucleus may be partly or wholly surrounded by the fine granular secretion without showing any sign of being affected. Neither mitochondria nor Golgi bodies seem to show any change in form, size, polarity, or distribution in the region of the cell peripheral to the zone of secretion. Mitochondria in the form of granules, rods, or even filaments are frequently present in this zone (figs. 12, 24). They may be quite numerous in zones which show signs of recent origin. Golgi bodies have not been seen to be present among the secretory material. There seems to be a sharp line marking off the territory of Golgi bodies from the zone. Just next to the outer border of the zone of secretion the Golgi bodies are decidedly more crowded than in the peripheral region of the cell, as if they have been pushed back by the spreading of the zone of fine granular secretion (fig. 20).

In studying the sections of glands after thirty-six hours of forced activity I found, in many of the gland cells, numerous basophilic bodies in the cytoplasm just peripheral to the nucleus. I have come to the conclusion that they are migrated nucleoli for the following reasons: 1) they are of the same size and take on the same stains as the nucleoli within the nucleus; 2) they are present in glands fixed in osmic-acid mixtures as well as in fluids poor for cytoplasmic inclusions, such as Gilson's and Bouin's (figs. 15 and 16); 3) they can be definitely distinguished from the mitochondrial elements when these are properly prepared in the same section (fig. 14); and, 4) it is not at all uncommon to find them in such positions just at the junction between the nucleus and the cytoplasm where the nuclear membrane is indistinct or absent, thus indicating that they are probably in the process of migration (fig. 14). These basophilic bodies, to which I shall from now on refer as migrated nucleoli, were later found to be present in normally active glands as well as in glands forced to continuous activity for periods shorter or longer than thirty-six hours. But they are most abundant in glands of this period, hence their first discovery was made here. I have not been able to tell whether there is an appreciable decrease in the number of nucleoli within the nuclei in cells which present large numbers of migrated nucleoli in the cytoplasm, on account of the irregularity in the distribution of the nucleoli inside the nucleus already mentioned.

Another characteristic phenomenon frequently met with in glands under stressed activity for thirty-six hours may be represented by figures 13 and 26. In figure 13 we find, at the level of the nucleus, numerous bodies out in the cytoplasm. While most of these bodies are lightly stained as the silk substance in the lumen of the gland, there are two solid dark bodies and one compound body which has a dark center and a light peripheral layer. The lowermost small, dark body is of the same size as the nucleoli in the nucleus. If this body should represent a recently migrated nucleolus, and I have reason to believe that it does, then we see a gradual transition from a migrated nucleolus to the fully formed secretory inclusion, that is, the migrated nucleolus grows in size and is transformed into the secretory body by a chemical change which begins from the periphery. This point is particularly well illustrated in figure 26.

In glands forced to continuous activity for forty-eight and seventy-two hours numerous lightly stained secretory inclusions are frequently seen near the inner border of the cell. These are generally so arranged that the smallest ones are always on the inside next to the lumen, while the largest bodies are peripheral. In case they are very numerous (they are never as numerous or as large as those found in glands following the cold-room treatment), the peripheral large ones nearly always show a close connection with the nucleus. Neither Golgi bodies nor mitochondria have been observed to be in any way associated with any of these inclusions. In the case of mitochondria, it is questionable whether they might not play a minor rôle in silk secretion. In figure 25 we see that, in the inner border of the gland cell, portions or granules of mitochondrial elements are hardly distinguishable from the small secretory granules in the same region.

Any and all of the various phases of activity may be found in glands forced to continuous activity for longer periods; but, aside from the fact that the glands decrease progressively in size with the increase in length of the time of forced activity, no new phenomena have been found. By the end of 200 hours, the larvae have generally decreased to one-third or even to one-fourth of their original size; the fat-bodies, which form a considerable bulk of the internal contents of a normally active larva, have almost entirely disappeared; and the spinning glands are now only about one-half or one-third as large as the glands of normally active larvae of the same size as these larvae were at the beginning of the experiment. On studying the sections of these glands, however, one finds them little different from those of the smaller normally active larvae.

Glands under cold-room treatment. Perhaps the most unique and striking pictures are offered by glands that have been under cold-room treatment. Here, the striking change to be described is true not merely for 10, 20, or 30 per cent of the glands as in the foregoing cases, but for 80, 90, and even as high as 95 per cent of the glands. Glands in cold-room for twelve hours present numerous but rather small secretory inclusions which crowd together in the inner portion of the cell. Here again, the smallest ones are always on the side next to the lumen. With the increase in length of time in the cold-room, the secretory inclusions increase both in number and in size. The larger peripheral ones are generally in close association with the nucleus. In looking over the section of these glands, one is impressed by the fact that the secretory inclusions apparently grow at the expense of the nucleus. Figure 21, for instance, shows that portions of the nucleus are on the verge of disappearance. In some of the glands which have been in cold-room for seven days, the entire cross-section of a gland may be almost completely filled with vacuoles and inclusions and hardly a trace of the nuclei is left.

The Golgi bodies are very difficult to impregnate in the glands following the cold-room treatment; even after prolonged impregnation they are never as dark as those in the normally active glands. But where they are distinct enough for study, no connection between Golgi bodies and the secretory inclusions has been detected (fig. 21).

Glands of ligated larvae. Still a different picture of the secretory activity is offered by glands of larvae ligated for twenty-four hours. Figure 16 may be used as a typical example. At the level of the nucleus as well as peripheral to it, there are numerous deeply stained bodies of all forms and sizes scattered in the cytoplasm of the cell. The numerous small bodies which are of the same size and take on the same stains as the nucleoli in the nucleus are probably recently migrated nucleoli. The next larger bodies in the form of rings are actually spheres, each with what appears to be a fatty center and a peripheral layer of basophilic substance, the fat having been dissolved away. In glands fixed in osmic-acid mixtures, the fatty constituent of these compound bodies is preserved and can be readily identified. The two largest bodies are basophilic throughout. It thus seems that the migrated nucleoli synthesize a lipoidal material in the center as they grow, this lipoidal material being finally transformed into a non-soluble substance. In a number of glands similarly treated, besides a small number of migrated nucleoli scattered in the cytoplasm in the vicinity of the nucleus, many good-sized basophilic bodies and a few lightly stained secretory bodies of like sizes are present. These lightly stained bodies are more likely to be near the inner border of the cell. This would fall in line with our expectation, if we assume that these bodies have come from the purely basophilic bodies and therefore represent a later stage in the development of the nucleolus into the secretory substance.

Finally, a fact worth mentioning is the frequent occurrence of very active cells and inactive cells in the same cross-section of a gland. This would mean that each gland cell is an independent physiological unit by itself.

Considerations

From the facts thus far presented we can reconstruct the complete history of the secretion of silk from the nucleoli: some of the nucleoli in the nucleus migrate out into the cytosome where they synthesize a fatty material in the center as they grow; this fatty material is gradually transformed into a non-soluble basophilic substance which is in turn changed into the secretion. The processes both in converting the fatty material into the non-soluble basophilic material as well as in converting the latter into the secretory substance in its final form, progress inwardly from the periphery of each body. Each secretory body is not to be regarded as a solid body in the living cell, but rather as a mass of semi-fluid secretory substance, which then separates into smaller and smaller masses and eventually into a fine, dispersed state just before it passes into the lumen of the gland. In the normally active gland this whole series of events moves on very rapidly in cycles, and as the supply probably exceeds the consumption, there is normally a constant reserve of the finely granular secretory substance evenly distributed in the cytoplasm. During excessive spinning the demand suddenly exceeds the normal rate of supply; consequently, the reserved secretory material in the cytoplasm is called upon. This physiological reaction may be so intense that the concentration process of the stored secretion exceeds, at certain times, its outflow into the lumen, thus the formation of a zone of fine granular secretion in the inner border of the cell. In the meantime, the secretory activity of the cell has greatly increased; hence most of the various phases of the secretory cycle have been discovered in glands after thirty-six hours of forced activity. My assumption that the secretory bodies break up into smaller and smaller bodies and eventually into a highly dispersed state is based upon the fact that whenever secretory inclusions are present in the cell, the smallest ones are invariably on the lumen side of the gland cell. Further, I have not observed, with the possible exception of glands kept in cold-room for seven days, any indication of an entire secretory body passing through the intima into the lumen. The breaking up of a large body or mass of semiliquid secretory material into a number of smaller masses is evidently an advantage in the migration of the secretory material from its place of origin toward the lumen.

A question arises as to why the compound bodies with a peripheral basophilic substance and a fatty center appear only in glands of larvae ligated for twenty-four hours. I can answer this question only by assuming that some unknown factor which retards the physiological process at this particular phase of secretory activity is in operation.

It has been noted that, in the case of larvae forced to spin continuously for as long as 200 hours, although they have decreased to one-third or even one-fourth of their original size, the nuclei of the spinning gland do not show any marked difference from those of the normal. On the other hand, in the case of larvae kept in cold-room for three to seven days, although they still retain their original size, the nuclei of the spinning gland have nearly disappeared with the increase, both in number and in size, of the secretory bodies. This remarkable difference, I believe, is due to the presence of a recovery process in the nucleus in the former case and the lack of such a process in the latter where the temperature is low. From this we may further infer that a relatively higher temperature is necessary in the recovery of the nucleus than is necessary in the secretory phenomenon.

We may now compare the results of the present study with those of the other investigators who have worked on the spinning glands of insects. Gilson ('90), working on the silk secretion in the silkworm, concluded:

> Les cellules épithéliales, formant la paroi des glandes séricigènes, élaborent une substance visqueuse complexe qui, dans certaines conditions, peut s'accumuler sous une forme visible dans leur cytoplasme et dans leur noyau. Le noyau prend donc part aussi à la production de cette substance.

Maziarski ('11), who made an extensive cytological study on the secretory phenomena in the spinning glands of the larvae of a number of butterflies and moths, said, in part, in his conclusion:

> Le noyau joue un rôle important dans les precessus sécrétoires; il participe directment à la formation du produit de sécrétion. C'est la substance nucléolaire qui donne la matériel pour les processus sécrétoires. Sélon l'intensité des processus fonctionnels, la substance nucléolaire est éliminée du noyau tantôt sous la forme de corps séparés, tantôt sous celle d'un liquide produit par la dissolution des nucléoles dans l'intérieur du noyau.

Marshall and Vorhies ('06), working on the spinning glands of Platyphylax larvae, suggested in their drawings that the nucleus might play some part in the secretory activity of the cell. Later, Vorhies ('08) definitely suggested that the nucleoli might play a rôle in secretion. Nakahara ('17) came to the conclusion in his studies on the larvae of the common cabbage butterfly, Pieris rapae, and those of a large caddis fly, Neuronia postica, that "in the silk-gland cells of insects studied, a portion of the nucleoli migrates into the cell body, and it forms at least a part of the secretion products of the cell." Kinney ('26) described, in the silk gland of Hyphantria cunea, that the nuclear bodies may be pinched off from the nucleoloid bodies and pass out into the cytoplasm where they undergo further modification and later become discharged into the lumen. She also suggested that the mitochondria might play some part in secretion. Noel and Paillot ('27, '28) described and figured, in their careful studies on the silk glands of Bombyx mori, the migration of the acidophile bodies (nucleoli) into the cytoplasm, where these bodies gradually take up the basic stains and later on are carried away by the streaming of the cytoplasm. They think that the silk substance is "d'une sorte de collaboration de noyau et du protoplasme." In regard to the rôle of mitochondria in secretion, they said: "Le rôle du chondriome, à en juger du seul point de vue morphologique, est de beaucoup moins important que celui joué par les nucléoles émigrés dans le protoplasme." The actual demonstration by vital staining of the migration of nucleoli into the cytoplasm by Dehorne and Hosselet ('28) has already been referred to. They described, in the spinning glands of a number of the Phryganid caddis worms, the different elaborate processes by which the emigrated 'nucleo-rouge' bodies give rise to mitochondria, which then elaborate the secretory substance. Finally, the work of Beams and Wu ('29), on the spinning glands of Platyphylax larvae, gave almost identical results, in regard to the rôles of nucleoli and Golgi bodies in secretion, as those presented in this paper. No one who has carefully reviewed the works on the spinning glands of insects can fail to see this one outstanding agreement among all the authors, namely, the important rôle played by the nucleus in secretion.

While the rôle played by the nucleus in secretion in the spinning glands of insects seems to have been definitely established, the evidence of a similar rôle of the nucleus elsewhere is rather meager. The only worker requiring our attention is that of Schreiner ('15, '16), who shows that, in the gland cells of the epidermis in Myxine, fat-drops and mucin bodies are derived from fragments of nucleoli that have migrated out into the cytoplasm of the cell.

Bishop ('22, '23), working on the histolysis of the fat-body cells of the honeybee, finds that large numbers of basophilic granules, possibly of the nature of nucleoli, migrate from the nucleus into the cytoplasm, where they enlarge at the expense of the latter to form the albuminoid globules, which are eventually released into the blood upon dissolution of the cell wall. This work of Bishop's may lead some to regard the phenomenal changes in the spinning glands of Galleria following cold-room treatment as also signs of fatty degeneration. That such is unlikely to be the case may be evidenced by the following facts: 1) larvae in cold-room for three days regain their activity in five to ten minutes at normal room temperatures; 2) the amount of silk substance in the gland lumen increases with the length of time during which the larvae are kept in the cold-room; and, 3) a similar phenomenon, namely, the presence of large numbers of inclusions in the cell, is frequently encountered with in normally active glands that have not been subjected to cold-room treatment. Furthermore, the histolysis of the insect fat-body is actively brought about, as shown by Bishop, by the cell through the agencies of nucleoli. If such is the case, then I see no serious objection to the view that the nucleoli take part in the elaboration of silk substance from some source material which also comes indirectly from the fat-bodies.

The mitochondrial theory of secretion has received much support from the works of Regaud ('09), Hoven ('10, '11), and others. According to this theory, the mitochondria, either by budding or by fragmentation, give rise to secretory granules which then enlarge to form the secretion. Very recently, Ma ('28), by means of differential stains, has succeeded in staining small granules which are still attached to the mitochondrial filaments in the same color as that of the large secretory granules, but different from the color of the mitochondria themselves. Nevertheless, Ma expresses the opinion that mitochondria alone cannot account for all the secretion and that other cell components, such as the ergastoplasm and the Golgi elements, might jointly contribute to the formation of secretion.

The rôle of the Golgi apparatus in secretion, through the extensive works of Nassonov ('23, '24), Bowen ('23, '24, 26), and others, has found favor in the eyes of many cytologists of to-day. Bowen ('26) is of the opinion that the Golgi apparatus plays by far the most important rôle in secretion among the cell components, and thinks that this might be a universal phenomenon.

It is not the aim of the author in this paper to discuss broadly the various theories of secretion, but to merely record the results of his studies on the spinning glands of Galleria concerning the respective rôles played by the nucleus, the Golgi bodies, and the mitochondria in secretion—results which agree, in all essentials, with those of the previous investigators working on similar subjects, namely: the nucleus plays a direct and important rôle, the mitochondria play a doubtful and at most a minor rôle, while the Golgi bodies play, if any rôle at all, a rôle which is not evidenced by their morphological behavior. In the author's opinion, the secretory substance in the spinning glands of Galleria is formed, in the words of Noel and Paillot, "d'une sorte de collaboration de noyau et du protoplasme."

The author is rather in doubt on the accuracy of observation of Gilson, who describes and figures the presence of secretory bodies inside the nucleus, and that of Maziarski, who says that the nuclear substance may be eliminated from the nucleus in the form of a liquid product as a result of the dissolution of nucleoli in the interior of the nucleus. If such were true in the forms studied by these authors, it is certainly not the case in the spinning glands of the wax-moth larva. The elaborately branching character of the nuclei in the spinning gland and the frequent close association of the secretory bodies with the nucleus make it possible to have, in the prepared sections, a condition with a secretory body surrounded on all sides by the nucleus, although the secretory body is, in reality, on the outside of the nucleus (fig. 20). The author is in agreement with Nakahara ('17), who has expressed his doubt concerning the statement of Maziarski cited above; for the nucleus and the cytosome are apparently two different physiological environments, and the subsequent changes of the nucleoli are possible only in the environment of the cytoplasm. The observation of Dehorne and Hosselet ('28) on the direct transformation of the migrated nucleoli into mitochondria is a very interesting one and compares well with that of Schreiner ('15), who finds a similar thing in the subcutaneous fat-cells of Myxine. The author is not in a position to pass judgment on this point beyond stating that such a condition had not been observed by him in the spinning glands of Galleria, although in sections where the mitochondria have undergone partial contraction, one may frequently find a graded series from the migrated nucleoli, through the somewhat smaller granular and rod-like mitochondria, to mitochondria of the long filamentous type.

SUMMARY

The points which have been brought out as a result of this study and which probably hold true, at least in part, for the spinning glands of the other lepidopterous insects are as follows:

1. In the spinning gland of Galleria mellonella three regions are distinguishable: an anterior conductive portion, a middle reservoir portion, and posterior secretory portion.

2. The nuclei of the cells of the conductive portion are typically unbranched, while those of the reservoir and secretory portions are highly branched.

3. Two types of bodies are recognized in the nucleus of the conductive portion: a large ovoid, basophilic body, comparable to the 'nucleoloid body' of Kinney, and numerous small chromatin granules. Two types of bodies are also distinguishable in the nucleus of the reservoir and secretory portions: the small chromatin granules and the larger but less numerous nucleoli which are somewhat irregular in form, size, and in distribution.

4. The network structure between the spirocircular filaments of the intima of the reservoir and secretory portions

is probably a mere coagulation phenomenon rather than actual supporting structures, as thought by Gilson ('90).

5. The silk substance in the gland lumen differs in the different portions of the gland. In the secretory portion only a lightly stained oxyphilic substance is present. In the reservoir portion two substances are present in the lumen: a central-core substance which is basophilic and highly viscous in the living state and a peripheral-layer substance which possesses the same viscosity and staining properties as does the substance found in the secretory portion. This central-core substance may be present in the anterior end of the secretory portion as well. In the conductive portion only the basophilic substance is present.

6. The middle portion of the gland, besides serving as a reservoir, secretes, though in a minor degree, a substance similar to that of the secretory portion.

7. The peripheral-layer substance in the reservoir portion is seemingly of the same chemical nature as that found in the secretory portion. Both represent the condition of recently secreted stuff which gradually transforms into the basophilic silk substance of the reservoir and the conductive portions.

8. Spinning, in Galleria, begins shortly after hatching and continues throughout the larval life of the insect.

9. The gland cells secrete continuously, irrespective of the act of spinning.

10. Each gland cell is an independent physiological unit.

11. The nucleus plays a direct and an important rôle in silk secretion by the migration of nucleoli into the cytoplasm where they synthesize a fatty material in the center as they grow in size; the fatty material is transformed into a non-soluble basophilic substance, which then changes into the secretory product in its final form. The processes of converting the fat into the non-soluble substance and of converting the latter into the secretory product in its final form progress inwardly from the periphery of each secretory body.

12. The secretory bodies or masses of secretory material break up into smaller and smaller masses and eventually into a fine dispersed state before their entrance into the lumen of the gland.

13. The mitochondria are granular in the cells of the conductive portion and filamentous in those of the reservoir and secretory portions. In the secretory portion they are orientated with long axis toward the lumen of the gland. Their rôle in silk secretion is negligible or at most a minor one.

14. The Golgi apparatus is in the form of discrete ring- and half-ring-shaped bodies and remains so during all stages of secretion. If it plays any rôle in silk secretion, the fact has not been detected by the author.

15. Under normal conditions, the loss of nucleoli by the nucleus in secretion is replaced through a recovery process of the nucleus. This recovery process requires a relatively higher temperature than does the pure secretory phenomenon. In case the temperature falls below a certain limit, secretory activity may go on at the expense of the nucleus.

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BIBLIOGRAPHY

BEAMS, H. W., AND WU, C. F. 1929 Cytological studies on the spinning glands of Platyphylax designatus Walker (Trichoptera): respective rôles played by the nucleus and the Golgi apparatus during secretion. Jour. Morph. and Physiol., vol. 47.

BISHOP, G. H. 1922 Cell metabolism in the insect fat-body. I. Cytological changes accompanying growth and histolysis of the fat-body of Apis mellifica. Jour. Morph., vol. 36.

——— 1923 Cell metabolism in the insect fat-body. II. A functional interpretation of the changes in the fat-body cells of the honey-bee. Jour. Morph., vol. 37.

BOWEN, R. H. 1923 The origin of secretory granules. Proc. Nat. Acad. Sci., vol. 9.

——— 1924 On a possible relation between the Golgi apparatus and the secretory granules. Am. Jour. Anat., vol. 33.

——— 1926 Studies on the Golgi apparatus in gland cells. I. Glands associated with the alimentary tract. Q. J. M. S., vol. 70.

——— 1926 II. Glands producing lipoidal secretions—the so-called skin glands. Ibid., vol. 70.

——— 1926 III. Lachrymal glands and glands of the male reproductive system. Ibid., vol. 70.

——— 1926 IV. A critique of the topography, structure, and functioa the Golgi apparatus in glandular tissue. Ibid., vol. 70.

CHORINE, V., ET KORVINE-KROUSKOVSKY, M. 1929 Sur l'immunisation de fragments du corps des chenilles de Galleria mellonella. C. Rend. Soc. Biol., T. 100.

COMSTOCK, J. H. 1924 An introduction to entomology. Ithaca, New York.

COWDRY, E. V. 1918 The mitochondrial constituents of protoplasm. Carnegie Institution of Washington, Publication no. 271.

DEHORNE, A., ET HOSSELET, C. 1928 Le nucléo-rouge dans les cellules sérici gènes des Phryganides donne naissance au chondriome. C. Rend. Soc. Biol., T. 99.

GILSON, G. 1890 La soie et les appareils séricigènes. I. Lepidoptères. La Cellule, T. 6.

——— 1896 La soie et les appareils séricigènes. II. Trichoptères. La Cellule, T. 10.

HELM, F. E. 1876 Über die Spinndrüsen der Lepidopteren. Zeit. f. wiss Zool., Bd. 26.

HENNEGUY, L. 1904 Les insectes. Paris.

HOVEN, H. 1910 Contribution à l'étude du fonctionnement des cellules glandulaires. Anat. Anz., Bd. 37.

——— 1911 Du rôle du chondriome dans l'élaboration des produits de sécrétion de la glande mammaire. Anat. Anz., Bd. 39.

KINNEY, E. 1926 A cytological study of secretory phenomena in the silk gland of Hyphantria cunea. Biol. Bull., vol. 51.

KORSCHELT, E. 1896 Über die Struktur der Kerne in den Spinndrüsen der Raupen. Arch. f. mikroskop. Anat., Bd. 47.

——— 1897 Über den Bau der Kerne in den Spinndrüsen der Raupen. Arch f. mikroskop. Anat., Bd. 49.

LEE, B. 1928 Microtomist's vade-mecum, 9th ed. Philadelphia.

LUDFORD, R. J. 1922 The behavior of the Golgi bodies during nuclear division with special reference to amitosis in Dytiscus marginalis. Q. J. M. S., vol. 66.

——— 1926 Further modifications of the osmic acid methods in cytological technique. Jour. R. Micr. Soc.

MA, W. C. 1928 The relation of mitochondria and other cytoplasmic constituents to the formation of secretion granules. Am. Jour. Anat., vol. 41.

——— 1928 The relation of the mitochondria-Golgi complex to secretion. I. The pancreatic acinar cell of the toad. Chinese Jour. Physiol., vol. 2.

MA, W. C., LIM, AND LIU 1927 Changes in the Golgi apparatus of the gastric gland cells in relation to activity. Chinese Jour. Physiol., vol. 1.

MARSHALL, W. S., AND VORHIES, C. T. 1906 Cytological studies on the spinning glands of Platyphylax designatus Walker. Intern. Monatschr. f. Anat. u. Phys., Bd. 23.

MAZIARSKI, S. 1911 Recherches cytologiques sur les phénomènes sécrétoires dans les glandes filières des larves des Lépidoptères. Arch. f. Zellforsch., Bd. 6.

METALNIKOV, S. 1908 Recherches expérimentales sur les chenilles de Galleria mellonella. Arch. de Zool. Exp. et Gen., T. 8.

MEVES, F. 1897 Zur Struktur der Kerne in den Spinndrüsen der Raupe. Arch. f. mikroskop. Anat., Bd. 48.

NAKAHARA, W. 1917 Physiology of nucleoli as seen in silk gland cells of certain insects. Jour. Morph., vol. 29.

NASSONOV, D. 1923 Das Golgische Binnennetz und seine Beziehungen zu der Sekretion. Arch. f. mikroskop. Anat., Bd. 97.

——— 1924 Morphologische und experimentelle Untersuchungen an einigen Säugetierdrüsen. Arch. f. mikroskop. Anat., Bd. 100.

NOEL, R., ET PAILLOT, A. 1927 Sur la participation du noyau à la sécrétion dáns les cellules des tubes séricigènes chez le Bombyx du Murier. C. Rend. Soc. Biol., T. 97.

PAILLOT, A., ET NOEL, R. 1928 Recherches histophysiologiques sur le tissu adipeux des larves d'insectes. Bull. d'Histologie, T. 5.

——— 1928 Recherches histophysiologiques sur les glandes séricigènes et les pigments des larves d'insectes. Bull. d'Histologie, T. 5.

REGAUD, C. 1909 Participation du chondriome à la formation des grains de ségrégation dans les cellules des tubes contournes de rein. C. Rend. Soc. Biol., T. 66.

SCHREINER, K. E. 1915 Über Kern und Plasmaveränderungen in Fettzellen, etc. Anat. Anz., Bd. 48.

VORHIES, C. T. 1908 The development of the nuclei of the spinning gland cells of Platyphylax designatus Walker. Biol. Bull., vol. 15.

WILSON, E. B. 1925 The cell in development and inheritance, 3rd ed. New York.

PLATES

PLATE 1

EXPLANATION OF FIGURES

All the figures in this plate, with the exception of figures 1, 2, and 8, were made with the aid of a camera lucida (reduced one-half in reproduction).

1 Sketch of the spinning glands dissected out under binoculars. *h*, hypopharynx. × 7.

2 Schematic sketch of a gland in optical longitudinal section. *s*, silk thread. Note the relative thickness of the gland wall in the various regions as well as the distribution of the central-core substance in the lumen of the gland. × 15.

3 Cross-section of conductive portion of gland cut at level *A* in figure 2. Approximately × 240.

4 Same as figure 3, only greatly magnified. *c.i.*, chitinous layer of intima; *n*, nucleus; *nu*, 'nucleoloid body'; *s*, silk thread; *s.l.*, subcuticular layer. Approximately × 1000.

5 Cross-section of reservoir portion of gland cut at level *B* in figure 2. *c.w*, cell wall; *f.g.*, fat-globules; *n*, nucleus; *t*, trachea; central-core substance heavily stippled; peripheral layer lightly stippled. Approximately × 240.

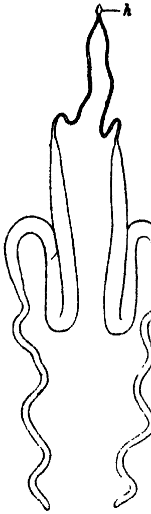
6 Half of a cross-section of reservoir portion cut at level *C* in figure 2. *c.c.*, central core of silk substance; *c.w.*, cell wall; *n*, nucleus; *p.l.*, peripheral layer of silk substance; *t*, trachea. Approximately × 240.

7 Cross-section of secretory portion of gland cut at level D in figure 2. *n*, nucleus; *t*, trachea. Silk substance in the lumen of this region is oxyphilic as the peripheral-layer substance of the reservoir portion. Approximately × 240.

8 Diagram showing the arrangement of cells in the anterior end of reservoir portion of gland; dotted area represents one gland cell.

9 Inner portion of part of a longitudinal section of secretory portion of gland. *f*, spirocircular filaments of intima. Approximately × 1500.

10 Tangential section of intima. *f*, spirocircular filaments of intima with the supposedly anastomosing strands of fibers between them. This structure of the intima is common to both the secretory and the reservoir portions of the gland. Approximately × 1500.



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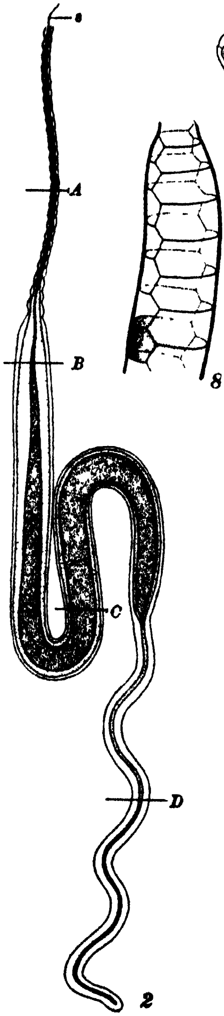

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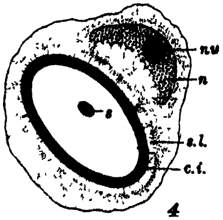

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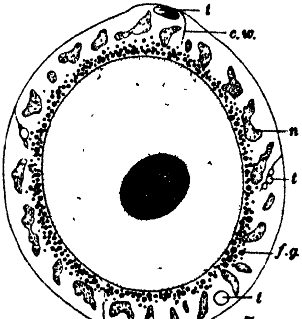

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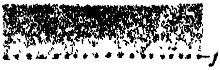

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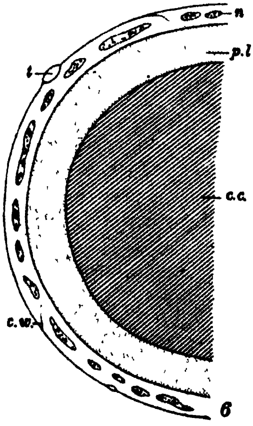

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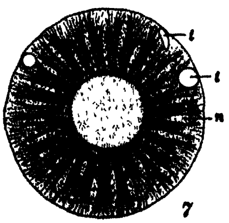

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PLATE 2

EXPLANATION OF FIGURES

All the figures in this plate were made with the aid of a camera lucida. Figures 11 to 17 were drawn at an initial magnification of approximately × 1000, while figure 18, at approximately × 1500. All except figures 15 and 18 are cross-sections of the secretory portion of glands under various experimental conditions (reduced one-half in reproduction).

11 Gland at normal activity; Mann's fixation; stain, iron haematoxylin (mitochondria having been completely destained from the section). *i*, intima; *p*, propria. Note the migrated nucleoli in the peripheral region of the cell.

12 Gland at end of thirty-six hours of forced activity; same fixation and stain as in figure 11. Mitochondria are in the contracted state, due to manipulation of gland in dissection. Note that the inner third of the cell is completely filled with a fine granular secretion. Also note the presence of mitochondria among the secretory substance. Two nucleoli have just migrated from the portion of the nucleus on the right-hand side. This state of activity makes its first appearance at the twelfth hour of forced spinning and may be present in any of the subsequent periods up to the eighth day, but is most prominent during the periods of twenty-four and thirty-six hours.

13 Gland at end of thirty-six hours of forced activity; same fixation and stain as in figure 11. The numerous bodies at the level of the nucleus display a definite transition from the dark migrated nucleoli to the lightly stained secretory inclusions. There are two solid dark bodies and one compound body which has a dark center and a light peripheral layer. The lowermost small, dark body is of the same size as the nucleoli in the nucleus and probably represents a recently migrated nucleolus. The compound body probably represents an intermediate stage between the migrated nucleolus and the lightly stained secretory bodies. This phase of activity was observed only in the thirty-six-hour period. It generally follows closely the phase seen in figure 12. Compare this figure with figure 26.

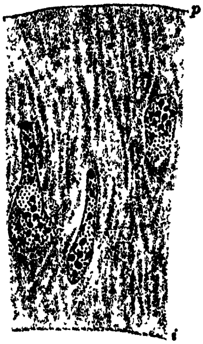
14 Gland after seventy-two hours of forced activity; Schridde's fixation; stain, iron haematoxylin. Note that the migrated nucleoli in the cytoplasm are hardly distinguishable from the nucleoli within the nucleus, but are easily distinguishable from the fine filamentous mitochondria. The details of the cytoplasm are left out in this drawing.

15 Part of cross-section of posterior end of the reservoir portion of gland after twenty-four hours of forced activity; Bouin's fixation; stain, iron haematoxylin. Note the similarity between the dark bodies in the periphery of the cell and the filamentous nucleoli within the nucleus. The vacuoles in the inner portion of the cell represent the remains of globules of fat that has been dissolved away. The smooth, roundish dark bodies in the middle of the cell probably represent an intermediate stage between the peripheral dark bodies and the fat-globules.

16 Gland of larva ligated and kept at room temperature for twenty-four hours; Bouin's fixation; stain, iron haematoxylin. Note that the smallest dark bodies out in the cytoplasm resemble most the nucleoli in the nucleus, and probably represent the recently emigrated nucleoli; the larger black rings are actually spheres each with what seems to be a fatty center and a peripheral layer of basophilic substance, the fat having been dissolved away; the two large solid black bodies probably represent a later stage where the fat has been changed into a non-soluble material before its final transformation into the silk substance. Note the disappearance of the peripheral portion of the nuclear membrane.

17 Gland from a well-fed larva; Mann's fixation; stain, safranin. *i*, intima. Compare this figure with figure 21.

18 Part of nucleus of secretory portion of gland, showing the different forms of nucleoli.



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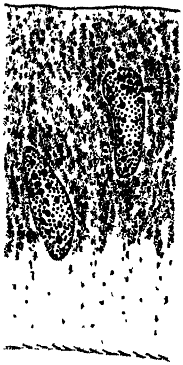

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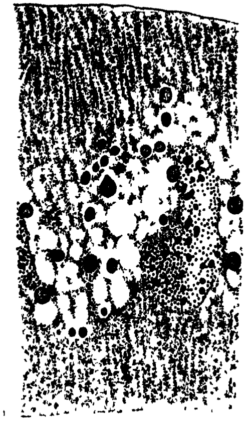

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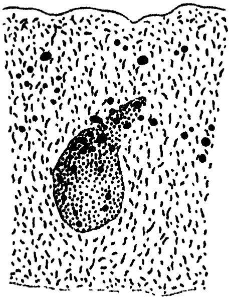

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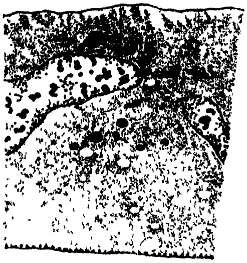

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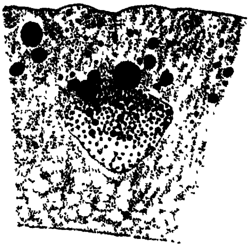

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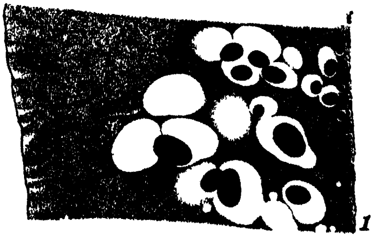

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PLATE 3

EXPLANATION OF FIGURES

All the figures in this plate were made with the aid of a camera lucida at an initial magnification of approximately × 1000. All except figures 23 and 27 are from the secretory portion of glands under various experimental conditions (reduced one-half in reproduction).

19 Normal gland; modified Mann-Kopsch technique. *i*, intima; *p*, propria. Golgi bodies are in the form of rings and half-rings, more or less evenly distributed throughout the cytoplasm. No nucleoli happen to be seen in this section because of their somewhat irregular distribution in the nucleus.

20 Gland at end of thirty-six hours of forced activity; modified Mann-Kopsch technique. Note the complete absence of Golgi bodies in the lower third of the cell which is occupied by the finely granular secretory material; also note the close association between the large secretory inclusion and the nucleus.

21 Gland of larva in cold-room for three days; modified Mann-Kopsch technique. The secretory inclusions have apparently grown at the expense of the nucleus. The vacuole around each secretory inclusion is not to be regarded as such in the living cell, but rather as having resulted from a shrinkage of the secretory substance following chemical treatment.

22 Normal gland; Schridde's fixation; stain, iron haematoxylin. Mitochondria are typically filamentous; where they are short rods or granules, these are probably only portions that have been severed from the rest of the filaments in sectioning.

23 Anterior end of reservoir portion of normal gland; modified Mann-Kopsch technique. *f.g.*, fat-globules. Mitochondria instead of Golgi bodies are impregnated. Note that the mitochondria are not in any way associated with the fat-globules.

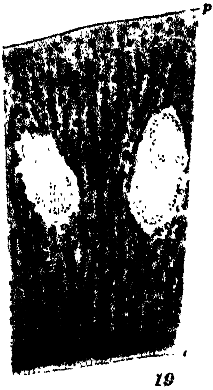
24 Inner portion of gland at end of seventy-two hours of forced activity; Schridde's fixation; stain, iron haematoxylin. *i*, intima. Some mitochondria are among the finely granular secretion. Compare this figure with figure 12.

25 Inner portion of gland similarly treated as that in figure 24. At the inner border of the gland, portions or granules of mitochondrial elements are hardly distinguishable from the small secretory granules in the same region.

26 Gland after thirty-six hours of forced activity, the section being taken at the level of the nucleus and more or less oblique; Mann's fixation; stain, iron haematoxylin. Three types of secretory bodies are present. The majority of the secretory inclusions take on the same stains as the silk substance inside the lumen. Two dark bodies in the lower left-hand corner are entirely basophilic, while the three on the right are compound structures, each with a basophilic center and an oxyphilic peripheral layer, particularly the one at *a*. These compound bodies represent, in my estimation, an intermediate stage between the purely basophilic and the purely oxyphilic bodies.

27 Reservoir portion of gland in cold-room for three days; modified Mann-Kopsch technique. *i*, intima. The secretory inclusions are the same as those found in the secretory portion of the gland.

28 Portion of intima and lumen of gland in cold-room for seven days; Champy's fixation; stain, safranin. *f*, filaments of intima; *s.s.*, secretory substance. The gland is so gorged with secretory substance that the silk substance in the lumen is directly continuous with that in the cell. Note that clear spaces instead of network of any kind are present between the filaments not occupied by secretory substance. These spaces have resulted from shrinkage undergone by the secretory substance following chemical treatment.



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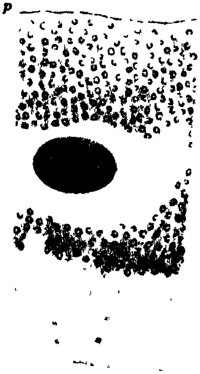

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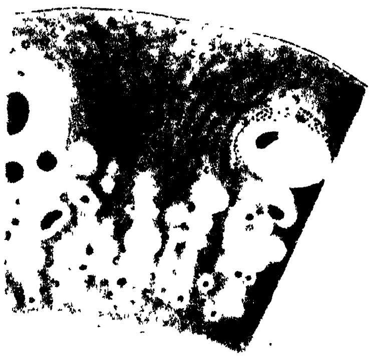

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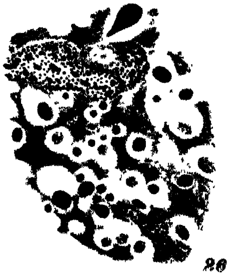

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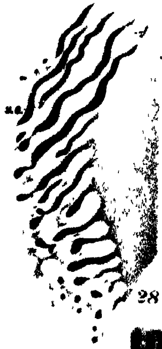

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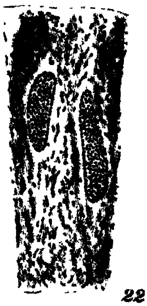

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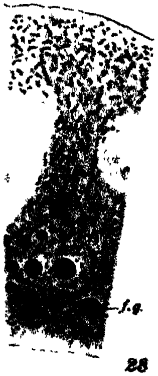

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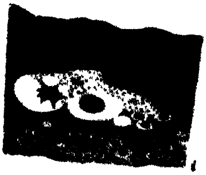

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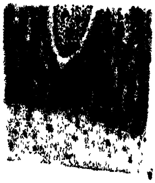

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THE HUMAN OVARIAN GERM CELLS[1]

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FOUR TEXT FIGURES AND FIVE PLATES (FORTY-TWO FIGURES)

AUTHORS' ABSTRACT

The germ cells in human embryonic ovaries arise by proliferations from the germinal epithelium These cells pass through the early maturation phases, including synizesis, beginning at about the third month. Four distinct periods may be distinguished in ovogenesis, each having its own peculiar characteristics: the early embryonic period from seven weeks to three months, the middle embryonic period from three to five months, the late embryonic period from five to seven months, and the adult period. The early embryonic period shows only growth and multiplicative phases; the middle embryonic period is distinguished by maturation phases, among which phases are interpolated which do not appear elsewhere in the species; the late embryonic period is characterized by phases similar to those of the adult male germ cells, and the adult period by the omission of early maturation phases preliminary to the maturation divisions.

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INTRODUCTION

Considerable work has been done in recent years on ovogenesis in mammals, notably that of Winiwarter ('00) on the rabbit and man and, with Sainmont ('08), on the cat. Their conclusions have been verified in most essential details by other investigators on the cat as well as a number of rodents.

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[1] Aided by grants from the Committee for Research in Problems of Sex of the National Research Council and the Board of Research of this institution.

It has been found that the whole process of ovogenesis in mammals differs to a considerable extent from the same process in lower organisms. In this group ovogenesis begins with the first differentiation of the germ cells in the ovary of the embryo; in man, as early as three and one-half months, as will be pointed out below. The ova, which are formed in enormous numbers by proliferations from the germinal epithelium, pass through the early maturation phases, a few even reaching the first maturation division. This continues until a few days after birth, when wholesale degeneration sets in and ultimately these ova all disappear, the definitive ova arising by later proliferations from the germinal epithelium. Evidences in the mouse and rat indicate that these later-formed, definitive ova do not pass through the typical maturation phases, this process here being shortened and modified beyond recognition.

But little work has been done on ovogenesis in human germ cells. This has probably been due to two factors: first, to the idea that human chromosomes are very unsatisfactory material to work with, and, secondly, to the great difficulty of obtaining well-fixed tissues. Material placed in 10 per cent formalin two or three hours after the operation does not offer a promising field for cytological study, yet this seems to be the usual mode of procedure in hospitals where such material must be obtained. It has been shown in our work ('29) on the sex and somatic chromosomes in man, however, that before dissolution of the nuclear membrane the contents of the nucleus are not greatly altered by a delay of even one or two hours, while a delay of ten minutes is usually fatal to the chromosomes after the membrane has disappeared. Since ovogenesis and maturation are processes which do not eliminate the nuclear membrane until the final division phase, it is evident that much material will prove adequate for this study which would be worthless for chromosome differentiation.

The most important work on the maturation of human ovarian germ cells is that of Winiwarter ('00) from a seven months' foetus. He describes a type of maturation for these cells comparable with that of the male germ cells, ending, however, in resting or growth phases after the pachynema. At the same time he described ovogenesis in the rabbit in which the maturation phases occur immediately after birth, the deutobroch nucleus appearing just before that time. This work was followed by a description of the same processes in the cat (Winiwarter and Sainmont, '08), in which the maturation phases were found to be embryonic processes similar to those in the rabbit and in man.

The next important work on ovogenesis in mammals was that of Kingery ('17) on the white mouse. Here, too, maturation was established as a phase of embryonic development. He, however, carried his investigations on to sexual maturity and found that maturation phases ceased to appear shortly after birth and that in the ova in the sexually mature mouse these phases were entirely omitted. It was found that all the embryonic germ cells had disappeared also within a few days after birth.

Allen ('23) extended these investigations on the mouse, with special emphasis on ovogenesis through sexual maturity. He questioned the validity of the concept that all definitive ova are formed before birth and remain quiescent until the onset of sexual maturity calls them into activity again. He found that the definitive ova arise by proliferations from the germinal epithelium in a cyclical manner at each normal oestrous period.

The same type of development of the ova was encountered in the ferret and ferret-polecat hybrids by Robinson ('18). He further found that successive generations of ovarian follicles were formed throughout the whole period of life.

Embryonic maturation of the germ cells of the ovary in their earliest phases thus seems to be a characteristic of the mammals. Whether development of the germ cells in the mature individual will follow that outlined by Kingery for the mouse can only be decided by further investigations.

MATERIAL AND METHODS

The opportunity for studying the development of the ovarian germ cells was presented in the acquisition by the laboratory of two human embryos in a well-preserved condition. The first of these was an embryo of about three and one-half months (9 cm. C.R. length) which had been fixed in Bouin's solution, the ovary being dissected out first. The second one was slightly more than five and one-half months (19 cm. C.R. length) in age. The ovaries of this were dissected out and fixed in Flemming's solution. Both ovaries were sectioned at 7.5 μ and stained in iron hematoxylin. A much smaller embryo was also available for study. This was an embryo which had been badly fragmented in removal, resulting in the immediate fixation of some of the organs, among them the ovaries. These were fixed in Bouin's solution and sectioned at 7.5 μ, the staining being done with iron hematoxylin. The length of this embryo was estimated at G.L. 25 mm., making its age between seven and eight weeks.

In addition to this embryonic material, the ovaries of the mother of the embryo of 19 cm. were studied. These had been fixed in Helley's solution and sectioned by the celloidin method. The stains used for these sections were Delafield's hematoxylin and eosin. Later a considerable number of adult ovaries became available for study.

In each of these cases, with the exception of the embryo of 25 mm., considerable delay had occurred before the material was placed in the fixing fluids, the length of time elapsing being nearly two hours in one instance. As has already been pointed out, this delay does not have the same effect on cells before the loss of the nuclear membrane as is the case after this has occurred. The figures shown in the plates are careful representations of cells as they appear under a one-sixteenth oil-immersion lens. A comparison of them with figures taken from material fixed in short periods and stained in the same manner (Evans and Swezy, '29, pls. 2 to 4) shows that no loss has occurred by reason of delayed fixation in either clearness or distinctness of nuclear contents.

EMBRYO OF 9 CM. C.R. LENGTH

The ovary of the embryo of 25 mm. was a minute organ. The germ cells presented a few mitotic figures, but gave no indications of any progress toward maturation changes. Cells in the germinal epithelium showed many mitoses, and along with this activity the forming germ cells could be distinguished from the other epithelial cells by their slightly greater size and the clearness of the cytoplasm. Here, as in the older ovaries, as will be pointed out later, new germ cells arise both by proliferations from the germinal epithelium and by division of already existing germ cells.

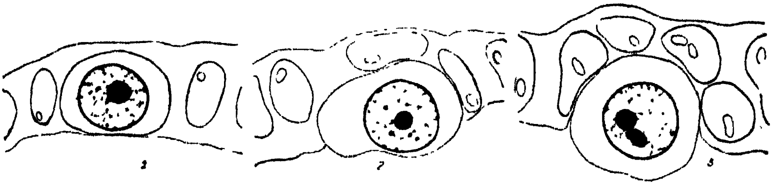
In the ovary of the embryo of 9 cm. it is at once seen that a very great advance has been made beyond the condition found in the younger embryo. The formation of new germ cells is proceeding actively and at the same time the older ova are passing through the early maturation phases. All stages of development are to be seen. These are not arranged in any perceivable sequence, but are scattered throughout the organ, though the larger cells are near the central regions. The division of oogonia, along with the series of maturation changes through which the nuclei pass, results in a cell formation that is remarkable for the variety of nuclear pictures which it presents. In this respect this ovary stands out in striking contrast to the simple form of the ovary of the earlier embryo.

Origin of ovum

It has already been pointed out that ova arise in two ways, one by proliferations from the germinal epithelium and the other by division of the germ cells already existing. In the ovary of the embryo of 9 cm. it is found that both of these methods are in full progress.

Ova arise from the germinal epithelium by a simple process. Any cell apparently is a potential ovum. The new ovum cannot be distinguished from the surrounding epithelial cells until both the cell and its nucleus undergo a slight enlargement as the beginning of the process of changing into a germ cell, the cytoplasm becoming clear and the nucleus more or less rounded at the same time (text fig. 1). When it has thus become clearly differentiated from the other epithelial cells, the ovum begins to move out from the germinal epithelium (text figs. 2 and 3) and comes to lie in the region immediately below, surrounded by other ova and connective-tissue cells. The number of ova arising in this manner is less in the ovary of this embryo than the number arising by division of preexisting ova.

Pratt and Long ('17) found that in the rat embryo of twelve to fifteen days the oogonia were actively dividing, but by the sixteenth day this had ceased and no more oogonial divisions occurred thereafter. In our work on the rat no mitotic figures have been found in the oogonia from the sixteenth day on to and through maturity. In the human ovary of 19 cm. no traces of oogonial divisions could be found. This process thus seems to be limited to the very early period of ovarian activity in the human as well as in the rat.



Text figs. 1 to 3 Figures of ova in germinal epithelium from ovary of embryo of 19 cm. The outer margin of epithelium is at the top. 1. Ovum becoming differentiated from epithelial cells. 2. Epithelium closing over germ cells. 3. Ovum leaving germinal epithelium.

Early maturation of ovum

The first indication of the imminence of maturation is a change in the nuclear contents. This appears as a distinct network with chromatin granules scattered through the interstices (fig. 1), among which are one or more nucleoli. It is difficult to say whether this change begins in cells which have just passed through the last oogonial division or in those which have arisen from the germinal epithelium. In the older ovary the latter, of course, is the case, the deutobroch nucleus usually appearing before the ovum has left the germinal epithelium. In the ovary of the embryo of 9 cm. the various cells present a confused mass that is very difficult to sort out into its special categories.

The network of the deutobroch nucleus breaks up into irregular threads and the small chromatin granules become gathered into large masses (figs. 2 and 3). The threads have the appearance of leptotene threads, but are fewer in number and soon disappear completely, leaving the nucleus filled with irregular masses and clouds of chromatin (figs. 4 and 5). These chromatin masses are finely granular, with short processes extending out from their borders. A condensation of the granules takes place, and as this proceeds chromatin bodies are formed which have some of the characteristics of the spermatocytic tetrads. As will be seen in figure 6, when this condensation takes place each mass of chromatin becomes a distinct dyad. In some cases these two moieties are quite separate, in other cases they remain connected by their ends and form rings (figs. 7 and 8). This condensation proceeds until each chromatin mass has assumed the form of a distinct chromosome, dual in nature, though this may be obscured by its position in some cases (fig. 9). Later these two parts seem to fuse again, though the shapes which they assume after this occurs often suggests the union of two smaller bodies (fig. 10). The figures here have a close resemblance to the tetrads of the first spermatocyte, but in nuclei where they can be counted there are forty-eight of these bodies. Whether this number is constant cannot be said. These bodies are similar to the prochromosomes which have been described in many insects, and hence they will be referred to in this paper by that designation.

The next change which these nuclei undergo is shown in figure 11. The nucleus becomes enlarged and the prochromosomes are massed at one side of the nucleus and close to the nuclear membrane, while the opposite side of the nucleus is clear. These prochromosomes then begin to lose their sharp outlines and become elongate and somewhat diffuse (fig. 12). As this continues it is found that each prochromosome is being resolved into a thread-like structure, thick and dark at first, but soon becoming more slender and less intense in its staining reactions (figs. 13 to 16). The threads thus formed have the appearance of the leptotene threads and their subsequent behavior confirms this impression. The leptonema does not fill the nucleus, but the larger part still retains the position at one side of the nucleus first assumed by the prochromosomes. Evidently some of the prochromosomes do not completely change into leptotene threads before the onset of synizesis, as a few bodies may still be distinguished in nearly every figure where the composition of the clump of chromatin is not too dense (figs. 17 and 18), though only one or two may be present.

By the time the formation of the leptotene threads is nearly completed, synizesis is well under way (figs. 18 and 19). The clumping of the chromatin mass is usually very dense and occupies a relatively small portion of the nucleus (fig. 20), the remainder being very clear in these cells. One or two large nucleoli may often be distinguished in this mass, one of which is generally found in a position near the nuclear membrane.

With the beginning of the pachynema (figs. 21 to 24), the chromosomes gradually emerge from the clump and spread out through the nucleus. They have the appearance of rather broad, granular threads or bands of varying lengths, with a more or less irregular outline.

Nuclei similar to the one shown in figure 23 are not abundant in this ovary, while the slightly earlier stages, such as those shown in figures 20 to 22, are fairly numerous. These are the final stages reached by the development of the germ cells in this material. The cells containing these nuclei are usually found near the central portion of the ovary and are conspicuous because of their size and the clearness of both nuclei and cytoplasm.

The centrosphere could not always be distinguished in the germ cells in this ovary. Where its presence could be detected (figs. 2 and 15) it was found to have the form of a crescent closely pressed to the wall of the nucleus. In the synaptotene stages it is found to abut on the nuclear membrane at a place opposite that of the chromatin clump within the nucleus. The different fixatives used in the two embryos account for the difference in the appearances of this structure in this and the ovary of the embryo of 19 cm.

EMBRYO OF 19 CM. C.R. LENGTH

The ovary of the embryo of this age differs in several respects from the one of 9 cm., as will be pointed out in detail in so far as the germ cells are concerned. The interior portion contains many large ova, some of which are surrounded by a single row of 'follicle' cells. Between these and the germinal epithelium are large masses of ova, varying in size, the smaller ones generally being found nearer the outer regions, though this is not an invariable rule. These show all developmental stages in the growth of the ovum and the different phases may be rather easily followed.

Origin of ovum

Unlike conditions in the two younger embryos, new ova in the embryo of this age arise mainly by proliferations from the germinal epithelium. A few mitotic figures may be found, but the number of these is negligible. The formation of new ova follows the same method already outlined in the discussion of the younger embryo (figs. 1 to 3). This process occurs abundantly in all sections of the ovary and the migration of the ovum from the germinal epithelium to the cortex below may be easily followed throughout all its stages.

In comparing this ovary with the one from the 9-cm. embryo, it is evident that the older ova are near the interior and the younger, newly forming ones, which are smaller, are near the margin. In the younger ovary this was not demonstrable, as the division of oogonia in all parts resulted in an intermixture of the small cells produced by division and those showing maturation changes.

Early maturation of the ovum

The appearance presented by a section of this ovary differs very markedly from that of the embryo of 9 cm. The astonishing variety of nuclear pictures found in the earlier embryo has to a large extent disappeared. Along with this, a clearer stratification of the cell categories has become evident. In the zone immediately below the germinal epithelium several rows of cells are composed of the newly formed germ cells only slightly larger than the original epithelial cells, but progressively enlarging as they recede from the epithelium. In the next zone the predominating cells are those which show the clumping of synizesis. The remaining regions are filled with cells which become greatly enlarged in many cases. There is a slight intermixture of cells in these zones, yet on the whole the different regions are rather clearly marked out, and this is especially true of the outer zones.

Degenerating cells are found most frequently in the central regions, though they appear elsewhere as well. Nuclei showing the block-like masses of chromatin, such as are abundant in the ovary of the embryo of 9 cm., are found here, sometimes in clumps of three or four, but these cells seem to be outside of the main line of development. They are probably cells remaining from the earlier ages.

The first stages in the early maturation of the ova are to be found in the outer zone just below the germinal epithelium, where a change begins to take place in the nuclear contents of the germ cells. The chromatin network becomes more evident with small chromatin granules lodged at the intersections, and one or more large nucleoli appear which take an intensely black stain with iron hematoxylin (fig. 25). This network is soon resolved into threads of varying lengths, the leptotene threads (figs. 26 and 27). These threads are finely granular and slender and usually do not stain very intensely. The entire nucleus is filled by them, and while the number cannot be counted, it is probable that the diploid number is present.

The leptotene threads gradually draw together and the next stage is produced (figs. 28 to 30). Here the entire mass of threads is found in a close, compact ball or clump at one side of the nucleus and close to the nuclear membrane. Extending out from its periphery the ends of some of the threads may be seen, sometimes in loops, or they may extend across the entire nucleus. In its final stage these ends have nearly all been withdrawn. A large nucleolus is usually evident in this mass near the point where it touches the nuclear membrane, outside of which the centrosphere is located.

Up to this stage the ovum has increased somewhat in size, but following this period the increase is more rapid both in nuclear as well as in cell size.

With the beginning of the pachytene stage (figs. 31 and 32) the chromatin mass unravels, during which process the chromosomes make their appearance. These are large and broad, rather diffuse in outline, the chromatin granules scattered irregularly through the paler-staining matrix. With their formation the entire nuclear space is again fully occupied. The large nucleolus becomes more evident, its shape rather irregular with a tendency toward a triangular form, slightly longer than wide. Often there are one or more smaller nucleoli, all of which take an intensely dark stain in iron hematoxylin.

The diplotene nucleus (figs. 33 to 35) shows the chromosomes condensed into more slender threads than in the previous stage. Their outline is still rather irregular, granules being freely scattered along their course. As these open out they often become twisted and in many of the threads a paired arrangement may be detected.

This stage gradually merges into the next (fig. 36), a transition phase between the diplotene and the dictyate which does not occur in this ovary. The diplotene threads or chromosomes become more slender and seem to lose their individuality to some extent and take on the appearance of a loose network, though part of the number still remain definite threads. The chromatin becomes massed in larger granules, but the nucleus as a whole shows a rather scanty amount of chromatin with a clear background, though this may be due not to a diminution in the actual amount of chromatin, but to the great enlargement of the nucleus which takes place at this time, the amount of chromatin remaining about the same, though probably losing much of its affinity for the stain. Two, sometimes three, large nucleoli are very conspicuous in all of this material. This is the final stage in this embryo when both the nucleus and the cell have reached their maximum size. All the large ova show this stage of nuclear development.

The centrosphere (yolk nucleus) becomes a striking object in these cells after the earliest stages are passed. It may be found in the leptonema, but is invariably present at the beginning of synizesis (fig. 28), where it appears as a crescent-shaped body lying close to the nuclear membrane near the point where the chromatin mass abuts on it. As the cell enlarges it enlarges also (figs. 33, 36), until it sometimes has a diameter of more than a third that of the nucleus. It is composed of a denser material which takes a slightly darker stain than the surrounding cytoplasm. Occasionally this darker mass is found in a zone which nearly or quite encircles the nucleus, and again two crescents may be present, each at opposite poles of the nucleus. The centrosomes cannot always be located with certainty in these stages, but when they do appear they have the form of two minute, intensely staining granules quite close to the nuclear membrane (fig. 31). It has not been possible to follow them in cells with the centrosphere encircling the nucleus or disposed in two crescents.

No evidences of true follicle formation have been noted in these ovaries. Many of the larger germ cells are surrounded by connective-tissue cells which form a complete investment. Other cells, the so-called indifferent cells, probably epithelial derivatives, are also found disposed in the same manner. These, however, are not strictly homologous with the follicle cells which form in the mature ovary. This has been most carefully traced in the rat, where they arise from the germinal epithelium with the ovum and are evidently sister cells. Kingery ('17) figures the same origin for the follicles in the mouse.

MATURE OVARY

Work on ovogenesis in the mature ovary is still in progress and will be dealt with very briefly here, a fuller discussion being reserved for a later communication. It has been found that new sex cells arise in a cyclical manner throughout mature life, the embryonic germ cells having disappeared some time between birth and the attainment of sexual maturity.

No ova were found which gave any indications of maturation phases. These are to be looked for in the ova which have just left the germinal epithelium and through the formation of the primary follicle—stages which are quite abundant in some of the material which has been studied. The type of nuclear structure (figs. 37 to 42) in these cells is similar to that in the germ cells in the ovary of the mature rat where maturation phases do not occur. A large, more or less spherical nucleolus occupies a prominent place in the picture, quite densely stained in the earlier stages, but becoming paler as the nuclear growth proceeds. A few threads forming a broken network with scattered clumps and clouds of chromatin make up the nuclear contents. In the younger nuclei, judging age by the distance from the germinal epithelium and the development of the follicle, the clumps of chromatin are more numerous and somewhat denser, with the threads less in evidence.

COMPARISONS

In general structure the ovaries of the three embryos vary but little except in size. It is in the germ cells that the most striking differences occur. In the ovary of the embryo of 25 mm. a condition is found which is strictly comparable with that encountered in the immature male gonad. Mitoses are abundant both in the germinal epithelium and in the cells which make up the cortex, as would be expected in a rapidly growing organ. Scattered through the cortex are large germinal cells, many of them in mitosis, but none of them giving any indication of approaching maturation in the nucleus. Conditions in the immature male gonad of the rat differ from this only in the fundamental structures which differentiate the testis from the ovary. The behavior of the germ cells and the germinal epithelium is the same in both cases.

It differs from the ovaries of the older embryos in that maturation changes have not yet begun.

The ovary of the embryo of 9 cm. presents a bewildering variety of ovarian cells, while the ovary of the embryo of 19 cm. has a comparatively narrow range of variations. This may be easily seen in comparing the figures in plates 1 to 4. These variations are found in the cells during the early part of the maturation phases. In the older embryo the deutobroch nucleus develops into the leptotene, followed by synizesis, while in the younger embryo the same nucleus develops by a long and elaborate process, in which there is a pairing of the chromatin masses into distinct prochromosomes which, in some cases at least, are forty-eight in number. These prochromosomes break up into leptotene threads at about the time synizesis begins, so that this phase is similar to that in the older embryo.

An occasional cell may be met with in the younger embryo in which the leptotene seems to be forming in the deutobroch nucleus, thus foreshadowing the change that is to take place at a later period. Also in the older embryo some cells are found in which the chromatin is arranged in blocks similar to the prochromosomes of the earlier stages. These are evidently cells left over from the earlier period.

In addition to this group of cells, dividing oogonia are to be found in both the younger ovaries, which differ in this respect from the one of 19 cm. where oogonial divisions have not been encountered. It is possible that the rare figures in mitosis which have been noted in the latter ovary are in reality dividing oogonia lingering on from the earlier age.

In Winiwarter's ('00) excellent paper on ovogenesis in human cells, his figures of maturation in a seven months' foetus agree in every respect with those of the 19-cm. embryo described in this paper. His figure 90, plate 7, which he notes as atypical, is evidently one of the prochromosome phases left over from the earlier developmental period. Such nuclei as this are occasionally seen in the 19-cm. embryo, as has just been noted, but they are quite plainly outside of the main line of development of that period.

The mature ovary differs very radically from the embryonic form, but structural changes need not be considered here. The enormous number of embryonic ova have all disappeared from the central regions, leaving only a narrow marginal zone which contains the germ cells which have the appearance of newly formed ova. These show all stages of the development of the primary follicle. The early maturation processes are non-existent in these cells, the differentiation of the ovum from the germinal epithelium being followed by a growth period.

The process of ovogenesis is thus seen to be divided into four periods, each with its own distinguishing characteristics: the early embryonic, the middle embryonic, the late embryonic, and the adult periods. The early embryonic period is marked by simple growth and multiplicative phases of the germ cells, the middle embryonic period is distinguished by maturation processes including phases which are found nowhere else in the species, the late embryonic period has dropped out these interpolated phases and the process of maturation is like that of the male germ cells, and the adult period is characterized by the absence of the usual maturation phases. Of these periods, only the early embryonic and the late embryonic resemble the process of spermatogenesis, the first in its simple growth and multiplicative phases and the second in its type of maturation. The other two developmental periods do not have their counterpart in the male gonad.

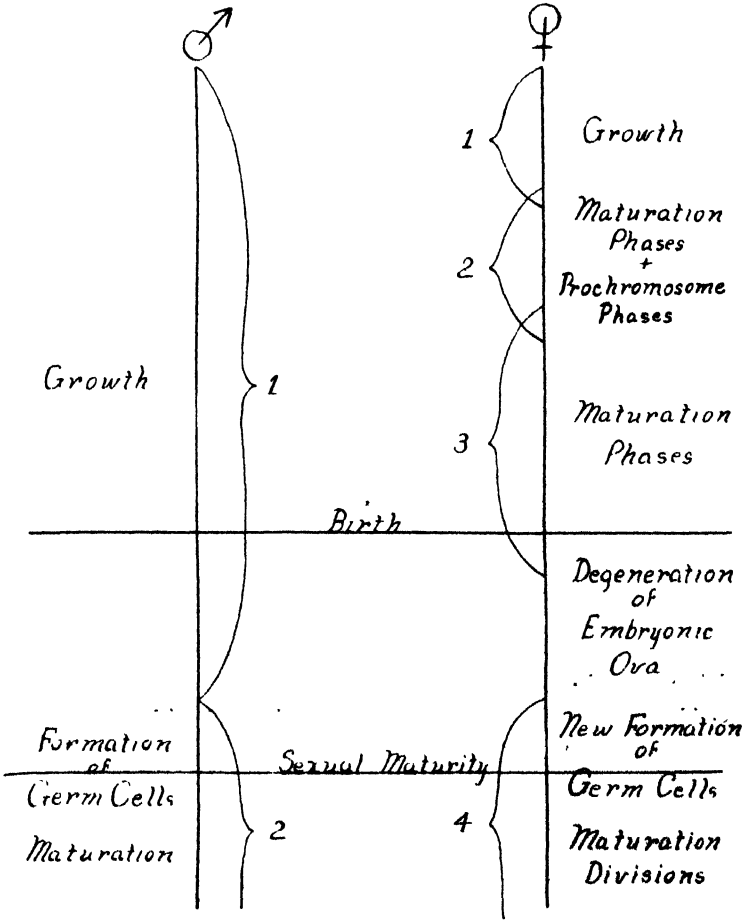
The cells of the embryonic periods, however, degenerate and ovogenesis in the definitive germ cells bears little resemblance to the process of spermatogenesis. A more complete series of ovaries in both embryonic and postpartum stages must be studied before it can be said that no further changes occur. Ovogenesis has been worked out quite fully in the rat (Swezy, '29) as well as by Kingery in the mouse, and by analogy with these the missing stages in the human material may be filled in with some degree of certainty. The development of the ova in both the rat and the mouse proceeds along nearly identical lines, the beginning in both rodents being similar to that of the late embryonic period as it has been described above, the more complicated phases of the middle embryonic period being absent in both cases. By the twentieth day postpartum the early maturation phases have been dropped out of the development of the ovum, and the adult period, in which these phases are not found, is similar to the same period described above for human material.

DISCUSSION

The processes of early maturation in the ovarian germ cells of the 19-cm. human embryo closely resemble those found in the maturation of the human adult male germ cells (Evans and Swezy, '29), the figures for each being identical save for size and the presence of the centrospheres. The agreement found in the behavior of the large nucleolus in the two germ-cell lines would suggest that this is the sex chromosome. This cannot be proved, however, since these ovarian cells do not proceed further in their development, judging by analogy with what we know as true for the embryonic ovarian germ cells in some other mammals as well as the structure of the mature ovary. The presence of the large centrosphere or yolk nucleus distinguishes these cells from the male germ cells, where a structure of this type does not occur. The most striking difference between the behavior of the germ cells in the two sexes, however, is that these maturation phases occur in the embryonic ova, while in the testis they do not occur until sexual maturity is reached. Also the spermatogonia continue to increase by division throughout adult life, while the oogonia do not divide after they have become differentiated from the germinal epithelium except for the final maturation divisions.

A further comparison may be made between the development of the two lines of germ cells. In the male germ cells the procedure is simple and consistent throughout, the period up to sexual maturity being passed in the slow processes of growth such as are characteristic of other organs of the body. In this it resembles the early embryonic period in the ovary. This is followed by an orderly method of maturation resulting in the production of spermatozoa with a haploid number of chromosomes. In contrast with this, the procedure followed by the female germ cells is revolutionary. At the age of three and one-half months in utero, the germ cells are found well advanced in the early processes of maturation, with the interpolation of additional phases such as are known chiefly in the insects. At the age of five and one-half months in utero, these additional phases have been dropped out and the processes of maturation conform quite closely to those followed by the adult male germ cells. When the adult ovary is reached, these germ cells are found to have completely disappeared, the new ova forming from the germinal epithelium. These pass through a period of growth without passing through the usual maturation phases, though the final maturation divisions produce oocytes with the haploid number of chromosomes. It is thus seen that the development of the two lines of definitive germ cells has only two points in common: they both arise from the germinal epithelium and each receives the haploid number of chromosomes in the final maturation divisions.

A graphic representation of this comparison of the behavior of the two lines of germ cells is given in text figure 4. A causal explanation of these differences can be looked for in the action of a maternal hormone or hormones which affect only the female germ cells. When the latter are in an immature stage, they respond by changes reminiscent of phases in the phylogenetic history of the species. These changes consist in the early maturation phases which in the younger embryos include the formation of prochromosomes. This action of maternal hormones on the embryonic ovary seems to be the rule throughout the Mammalia.



Text fig. 4 Diagram illustrating the course of development of the two lines of germ cells in man from the time of the differentiation of the gonads to sexual maturity.

The processes of ordinary somatic-cell division in the two sexes are identical in every respect as far as can be studied under the microscope, differing only in the size of the sex chromosomes and in the size of the two longest autosomes (Evans and Swezy, '29).

The same differences are found in the development of the two lines of sex cells in the rat (Swezy, '29), though the interpolation of other phases in the early maturation of the embryonic ova does not occur there. Kingery's ('17) work on the mouse indicates that development here shows the same differences in the two sexes as in the rat and in man.

It has been possible to follow out the development of the ovarian germ cells in the rat from birth to maturity. It is found that between five and twenty days postpartum the various maturation phases are gradually dropped out until in the ovary of the rat of twenty days no maturation phases are present. The ova from the earlier proliferations have all disappeared and new ones are continually being formed from the germinal epithelium. All the definitive ova in the mature rat are thus cells which have not passed through the early maturation phases. Studies on the embryonic and mature ovaries in man suggest the probability that when the gaps between the stages given here are filled, the course of development will be found to be quite similar to that in the rat.

The origin and method of development of these germ cells in the human embryonic ovary of 19 cm. are also similar to those of the embryonic ovary of the rabbit (Winiwarter, '00), the cat (Winiwarter and Sainmont, '08), and the mouse (Kingery, '17), as well as agreeing with what we have found in the rat. The recent work of Simkins ('28) on the origin of the sex cells in man shows that in younger and more complete series of ovaries than have been available for these studies "all the cells that go into the fundaments of the indifferent gonad are derived from the germinal epithelium."

The next stages in the development of these cells differ from those in the male germ cells in that the nucleus enters upon a resting phase, the dictyate, instead of forming the haploid group of chromosomes and dividing, agreeing in this respect also with the procedure in the cat and rodents. The dictyate has been termed the growth period, which, according to older embryologies may extend over many years. Since, however, the embryonic phases agree so closely with what we know to be the true course of development in some other mammals, it is probable that the later development is the same, i.e., that these embryonic ova degenerate after birth, as new ova are later formed from the germinal epithelium. In the ovary of the embryo of 19 cm. many ova are found to be degenerating and this degenerating process is a continuous one. As has already been noted, the structure of the mature ovary supports this conclusion.

In comparing the development of the human embryonic ovarian germ cells with those of the rabbit, cat, mouse, and rat, a signal difference is noted. In this group maturation phases do not appear until the latter part of pregnancy, the pachytene stage at about or after birth, the dictyate appearing one or two days later. In the human ovary the maturation processes begin at least as early as three and one-half months (9 cm. C.R. length), and at five months (19 cm. C.R.) the ovary is filled with ova showing most of the different maturation phases. A striking difference is to be noted, however. The phases which occur in these rodents and the cat are similar in every way to those which are present in the five and one-half months' embryo. The prochromosome formation which is so prominent in the human embryo of 9 cm. does not occur in these animals. This is probably accounted for by the fact that sexual differentiation of the gonads takes place at a relatively much earlier period in man than in the rat and perhaps other animals also. The prenatal development of the ovarian germ cells in man is greatly in advance of that of the cat and rodents.

The origin and the time of formation of the definitive germ cells have been a much discussed question, though the most careful recent work agrees in assigning this to the germinal epithelium, beginning with the onset of maturity. The general opinion on this subject has been summarized by Allen ('23) in a quotation from Pearl and Schoppe ('21) which follows: "It has been held as a basic biological doctrine that during the life of the individual there neither is nor can be any increase in the number of primary oocytes beyond those originally laid down when the ovary was formed." In the face of this, it is an established fact that in some of the lower vertebrates, fish (Ludwig, '74), amphibians (Gatenby, '16), and reptiles (Braun, '77), a regeneration of the ovary from the germinal epithelium occurs seasonally. The investigations of Kingery and Allen in the mouse and Robinson in the ferret have proved beyond doubt that in these animals the definitive germ cells do not come from ova laid down when the ovary was formed, but are new cells developed at the beginning of and during sexual maturity. Allen further found that these arise in cyclical waves coincident with each normal oestrous period. Simkins found that all the fundaments of the human ovary came from the germinal epithelium, though he concludes that the delivery of such cells ceases when the tunica albuginea is formed, as this prevents their ingress. Work on the rat shows that not only do the cells continue to proliferate from the germinal epithelium after the appearance of the tunica albuginea, but that this layer contributes to the formation of the follicle by supplying the investing theca. Ova have also been found proliferating from the germinal epithelium in the rat up to 398 days in a single instance and in three rats at 374 days. Robinson ('18) states that new ovarian follicles are formed throughout the whole period of life in the ferret and ferret-polecat hybrids. Papanicolaou's ('24) work on the guinea-pig shows that ova continue to arise in a cyclical manner from the germinal epithelium in that animal throughout the period of sexual maturity. This is probably the method followed by the Mammalia generally.

This is the first time that prochromosomes have been described from a mammal. These occur quite widely among the insects and are also known in plants. The fact that they do not occur in the adult of either sex does not lessen their significance in the embryonic germ cells. Each prochromosome is composed of mates which are distinct as they are first formed, later fusing into a single body which breaks up into a single thread. These threads then conjugate in the usual manner during synizesis, so that each chromosome resulting from synizesis is composed of four of the original elements appearing in the early prochromosome stage. This combination may give some support to the so-called 'anaphasis duality.' It further shows that the number of leptotene threads is the diploid forty-eight.

The formation of the prochromosomes is closely similar to that described for the beetle, Blaps, by Nonidez ('20), though he did not note a doubling of the units in each prochromosome. They are formed by the condensation of granular masses into block-like chromosomes with the diploid number present. These become polarized before unraveling into the leptotene threads in the same manner as that described for the human cells. In the beetle this is not followed by synizesis, but the leptotene threads change into the pachytene. His figures 65 to 76 have their counterpart in our figures 5 and 10 to 14.

The absence of maturation phases in the adult germ cells is in line with the development of the ova in the adult mouse as this has been carefully investigated by Kingery ('17) and in the cat, dog, and rat as that has been worked out in this laboratory. In the present instance it might be said that these phases occurred in the germinal epithelium, since a sufficient number of observations were not made on the cells of that region. This, of course, cannot be disproved until a more thorough search has been made. Maturation phases require a relatively long period in which to develop, and if these occurred in the germinal epithelium evidences of such changes should be found in great abundance, considering the number of cells which are produced, and no such evidences are forthcoming. All investigators on ovogenesis who have found ova in the germinal epithelium agree that the length of time in which ova may be distinguished in that position is very short. In the work on the rat and the mouse it has been shown that there was no possibility of these phases occurring in the germinal epithelium. It seems unlikely that it occurs in man, since the entire processes of ovogenesis and ovarian development appear to follow the same general lines.

Work on this paper had been completed when we had the good fortune to receive from Dr. G. W. Bartelmez, of the University of Chicago, the ovaries of two foetuses, one of 57 mm. C.R. length, and the other 93 mm. C.R. length. The smaller foetus (H 1207) had been fixed in formol and the larger one (H 607) in Zenker's solution. In the latter case the body cavity had been opened up five hours after it had been placed in the fixative, and was preserved in 80 per cent alcohol. Both were well preserved, particularly the second one, in which the germinal cells were very clear.

The ovary of the embryo of 57 mm. was somewhat advanced beyond the stage of development described above for the embryo of 25 mm. A few cells only showed that the first beginnings of the maturation phases were already present. In the ovary from the embryo of 93 mm. conditions similar to those described above for the 9-cm. embryo were found, including maturation phases with the formation of prochromosomes.

Two more ovaries were obtained, one from an embryo of about 35 mm. and the other of 70 mm. The condition in these confirmed in every detail the descriptions given above for the other embryos of similar age.

SUMMARY

1. The germ cells in human ovaries of 25 mm. and 9 cm. arise both by proliferations from the germinal epithelium and by the division of sex cells already formed. In an embryo of 19 cm. they arise only by proliferations from the germinal epithelium.

2. After leaving the germinal epithelium or after the last oogonial division, the nucleus of the ovum passes through the early maturation phases, including synizesis. In the

embryo of 9 cm. these phases include an elaborate process by means of which prochromosomes are formed and later changed into leptotene threads at the time of synizesis. Maturation in the older embryo is similar to that of the male germ cells, the prochromosomes being dropped out. These maturation phases do not occur in the ovary of the embryo of 25 mm.

3. True follicles are not formed in the embryonic ovary, the cells which make up the 'follicles' containing the larger ova being composed of 'indifferent' and connective-tissue cells.

4. In the adult ovary the embryonic germ cells have all disappeared. Maturation phases are not found in the development of adult ova.

5. Four distinct periods may be distinguished in ovogenesis, each having its own peculiar characteristics: the early embryonic, the middle embryonic, the late embryonic, and the adult periods. The early embryonic period shows only growth and multiplicative phases, the middle embryonic period is distinguished by maturation phases among which stages are interpolated which do not appear elsewhere in the species, the late embryonic period is characterized by maturation phases similar to those of the male germ cells, and the adult period by the omission of all maturation phases. The early embryonic period represented by the embryo of 25 mm. is comparable with the condition in the immature testis.

6. The origin and the course of development of the embryonic ova in man, with the exception of the middle embryonic period, are the same as in the rabbit, cat, mouse, ferret, and the rat. The prenatal development of the germ cells is much further advanced in man than is the case in these lower mammals. In man the maturation phases begin relatively much earlier and show phenomena which do not occur in the other mammals.

7. In the adult ovary the definitive germ cells arise by new proliferations from the germinal epithelium in a manner similar to the process in the cat, ferret, ferret-polecat hybrids, guinea-pig, mouse, and rat.

LITERATURE CITED

Allen, E. 1923 Ovogenesis during sexual maturity. Am. Jour. Anat., vol. 31, pp. 439–482.

Arai, H. 1920 On the postnatal development of the ovary (albino rat), with especial reference to the number of ova. Am. Jour. Anat., vol. 31, pp. 405–462.

Evans, H. M., and Swezy, O. 1928 A sex difference in chromosome lengths in the mammalia. Genetics, vol. 13, pp. 532–543

———— 1929 Human chromosomes; sex and somatic. Mem. Univ. Calif., vol. 9.

Gatenby, J. B. 1916 The transition of peritoneal epithelial cells into germ cells. Quart. Jour. Micr. Sci., vol. 61, pp. 275–300.

Kingery, H. M. 1917 Oogenesis in the white rat. Jour. Morph., vol. 30, pp. 261 316.

Nonidez, J. F. 1920 The meiotic phenomena in the spermatogenesis of Blaps with special reference to the X-complex. Jour. Morph., vol. 34, pp. 69–118.

Papanicolaou, G. N. 1924 Ovogenesis during sexual maturity as elucidated by experimental methods. Proc. Soc. Exp. Biol. Med., vol. 21, pp. 393–396.

Pearl, R., and Schoppe, W. F. 1921 Studies on the physiology of reproduction in the domestic fowl. Jour. Exp. Zoöl., vol. 34, pp. 101–118.

Pratt, B. H., and Long, J. A. 1917 The period of synapsis in the egg of the white rat, Mus norvegicus albinus. Jour. Morph., vol. 29, pp. 441–460.

Robinson, A. 1918 The formation, rupture, and closure of ovarian follicles in the ferret and ferret-polecat hybrids, and some associated phenomena. Proc. Roy. Soc. Edinburgh, vol. 52, pp. 303–362.

Simkins, C. S. 1928 Origin of the sex cells in man. Am. Jour. Anat., vol. 41, pp. 249–294.

Swezy, O. 1929 The ovarian chromosome cycle in a mixed rat strain. Jour. Morph. and Physiol., vol. 48, pp. 445–473.

Winiwarter, H. von 1900 Recherches sur l'ovogénèse et organogénèse de l'ovaire des mammifères (lapin et homme). Archiv. Biol., T. 17, pp. 33–199.

Winiwarter, H. von, and Sainmont, G. 1908 Nouvelles recherches sur l'ovogénèse et l'organogénèse de l'ovaire des mammifères (chat): Chapitre IV. Ovogénèse de la zone corticale primitive. Archiv. Biol., T. 24, pp. 165–276.

EXPLANATION OF PLATES

All figures were drawn with the aid of a camera lucida from human ovaries stained with iron hematoxylin unless otherwise stated. Magnification, 2560 diameters.

PLATE 1

EXPLANATION OF FIGURES

All figures were drawn from ovary of embryo of 9 cm. C.R. length.

1 Deutobroch nucleus.

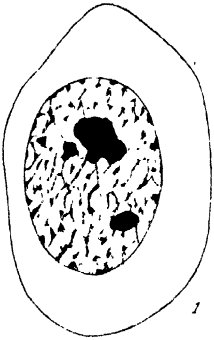
2, 3 Network of deutobroch nucleus breaking up and forming threads.

4, 5 Stages showing the threads of previous figures disappearing with the formation of clouds and clumps of chromatin.

6 Beginning of the formation of the dyads.

7, 8 Formation of the dyads.

9 Complete condensation of the chromatin masses into prochromosomes.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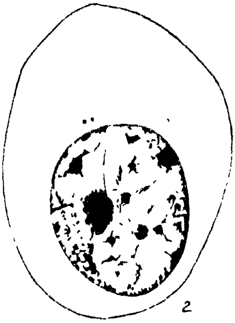
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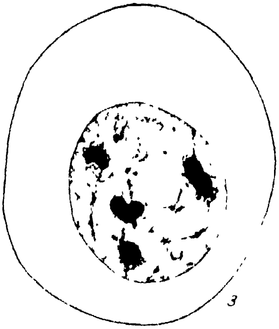
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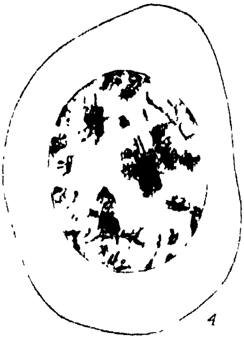
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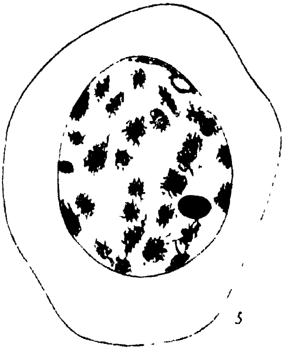
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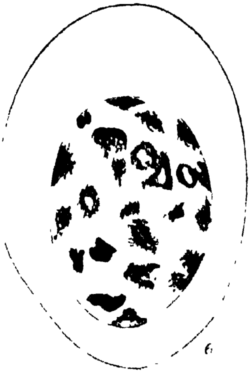
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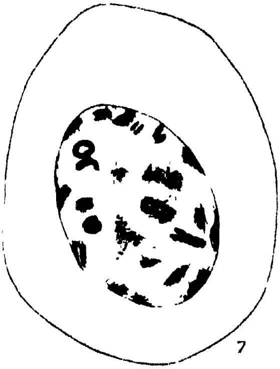
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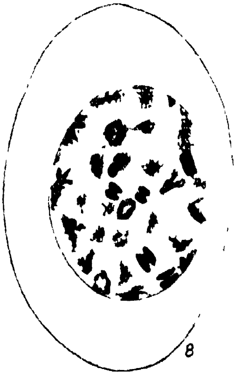
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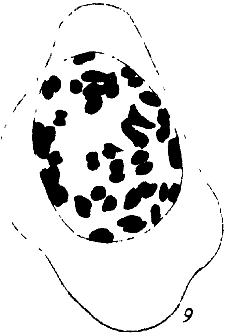
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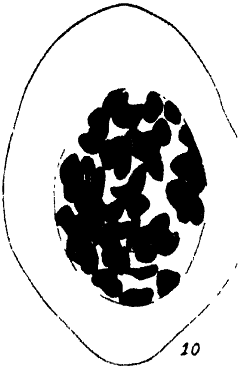
PLATE 2

EXPLANATION OF FIGURES

All figures drawn from ovary of embryo of 9 cm. C.R. length.

10 Fusion of the dyads into a single prochromosomes, forty-eight in number.
11 Polarization of the prochromosomes.
12 Beginning of the breaking up of the prochromosomes.
13 to 15 Stages in the changing of the prochromosomes into threads.
16 A further step in the same process. Only part of the nuclear contents drawn.
17 Beginning of synizesis.
18 Beginning of synizesis with almost complete transformation of the prochromosomes into leptotene threads.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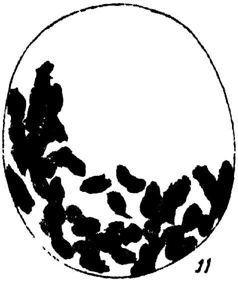
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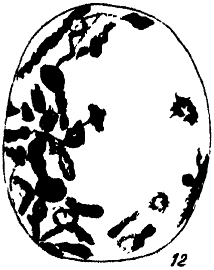
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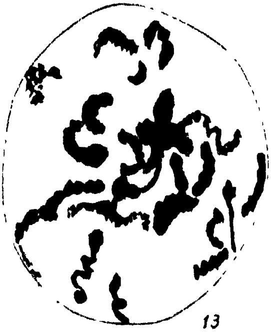
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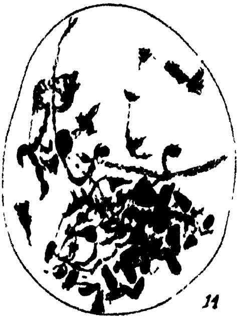
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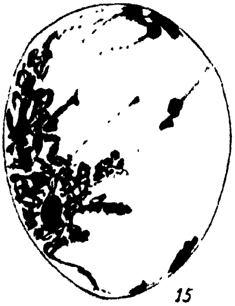
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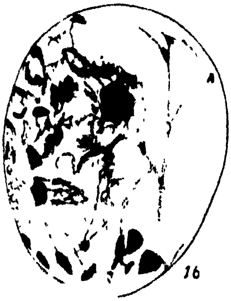
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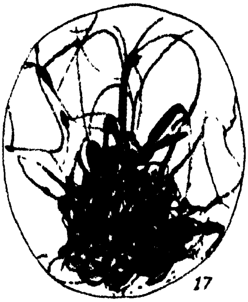
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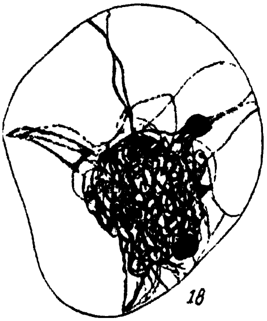
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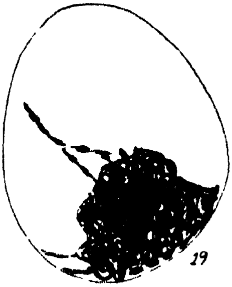
PLATE 3

EXPLANATION OF FIGURES

Figures 19 to 24 drawn from ovary of embryo of 9 cm., figures 25 to 27 drawn from ovary of embryo of 19 cm. C.R. length.

19, 20 Synizesis.
21 Beginning of the unraveling stage.
22 Pachynema.
23, 24 Later pachynema.
25 Deutobroch nucleus from the germinal epithelium.
26 Leptonema.
27 Later leptonema.



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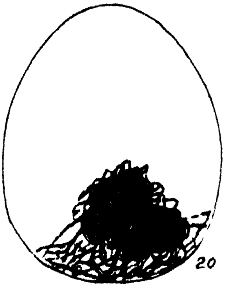

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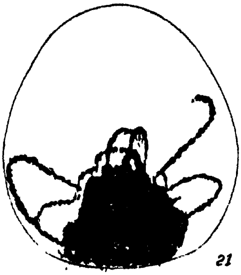

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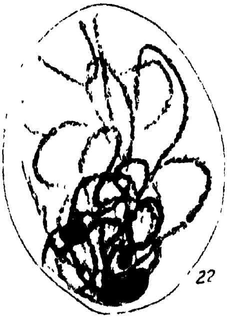

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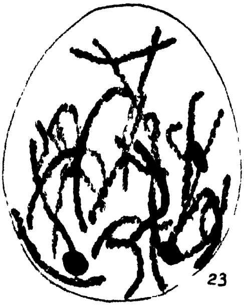

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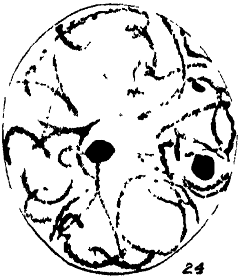

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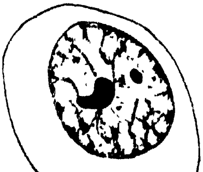

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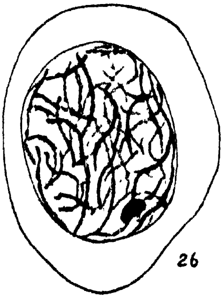

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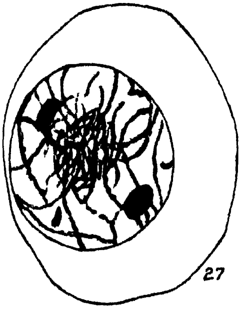

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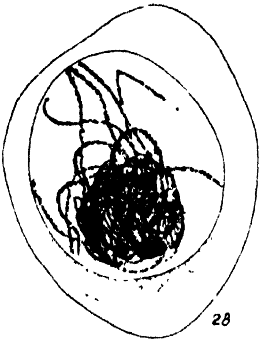
PLATE 4

EXPLANATION OF FIGURES

All figures drawn from ovary of embryo of 19 cm. C.R. length.

28 Early synizesis.
29, 30 Synizesis.
31 Beginning of the unraveling stage.
32, 33 Pachynema.
34, 35 Late pachynema.
36 Transitional stage between the pachynema and the dictyate.



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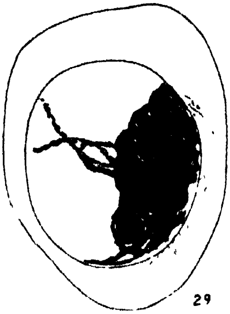

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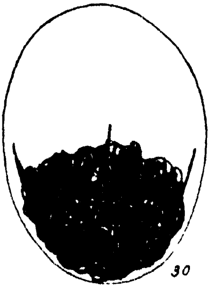

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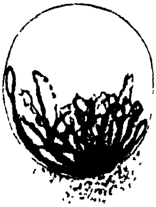

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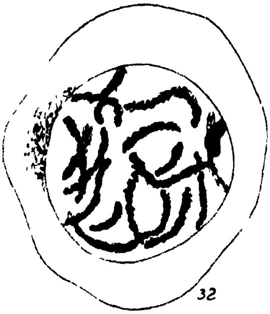

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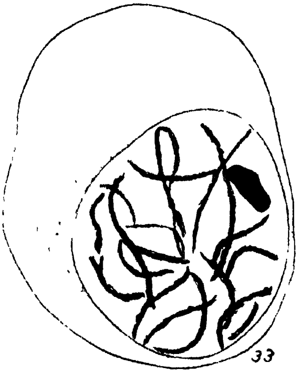

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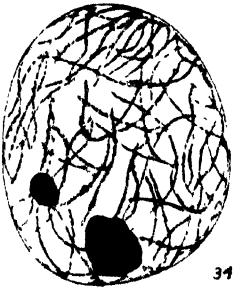

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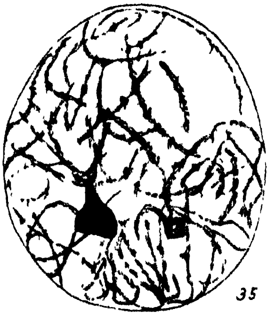

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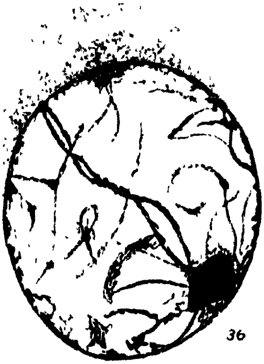

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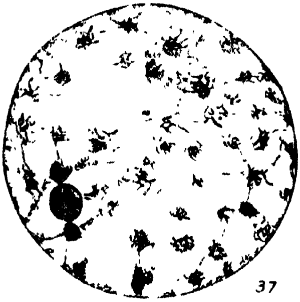
PLATE 5

EXPLANATION OF FIGURES

All figures drawn from adult ovary, fixed in Helley's solution and stained with Delafield's hematoxylin and eosin.

37 Nucleus from ovum with only few cells of follicles present.
38 to 40 Nuclei from ova with partial row of follicle cells.
41, 42 Nuclei from ova with primary follicle formed.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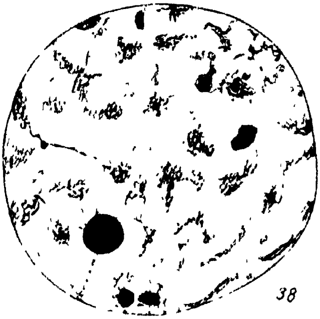
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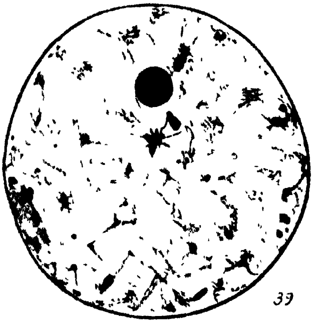
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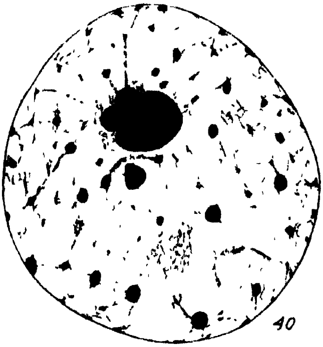
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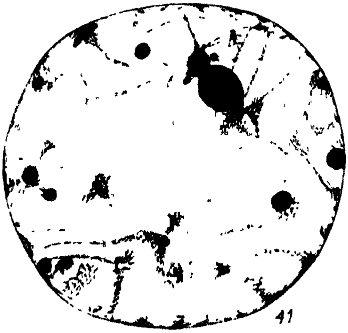
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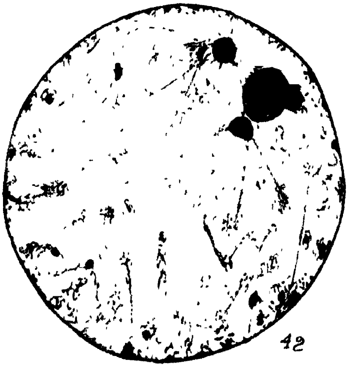
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THE EARLY DEVELOPMENT OF HAEMICHROMIS BIMACULATA, WITH SPECIAL REFERENCE TO FACTORS DETERMINING THE EMBRYONIC AXIS

ROBERT S. McEWEN
Department of Zoology, Oberlin College

TWO PLATES (TEN FIGURES)

AUTHOR'S ABSTRACT

Haemichromis bimaculata is a tropical teleost fish which will produce eggs practically throughout the year at intervals of from three to four weeks. These eggs are of suitable size and character for embryological study, the features of special interest so far discovered being as follows 1) The egg is oval, 2) it is attached to a substratum by its side, the blastopore being at one end, 3) the embryo always tends to develop along the side opposite that originally next to the substratum

Results obtained by reorienting the eggs previous to cleavage, and by centrifuging them, seem to show that the relation of the embryonic axis to the egg is determined either previous to laying or very soon afterward, possibly by the relation to the substratum, and is not subsequently affected by gravity or other known factors. There may be some tendency for the first cleavage plane and the sagittal plane of the embryo to coincide, but such coincidence is not at all constant or exact

INTRODUCTION

Though the problems of fish embryology have long been under investigation, there still remain unsolved questions in this field which invite attack. One difficulty, however, which besets workers in this, as in many other embryological studies, is the seasonal limitation imposed by the material. To such an extent is this true among the higher animals that it seems not unlikely that one of the chief reasons for the extensive and intensive labor upon one form, the chick, is due to the extreme convenience with which fertile eggs may be obtained practically throughout the year. There are certainly few vertebrates so readily available, and in the case of fish there have appeared to be none. At all events, investigations in this direction seem to have been confined entirely to forms with a relatively limited breeding season, such as Serranus, Fundulus, Ctenolabrus, Salmo, Coregonus, and others. There are, however, a great number of tropical fish which have never

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been carefully studied in this connection, and which are readily kept and bred in aquaria. It seemed likely, therefore, that if one of these varieties could be secured, possessing an egg of reasonable dimensions, and, if possible, with a frequently recurring breeding cycle, it might easily afford an ideal subject for year-round embryological study.

With this in mind, a number of inquiries were made which finally resulted, through a suggestion of Dr. W. N. Hess, in bringing the writer into touch with the late W. G. O'Brien, of the Tricker Nurseries at Independence, Ohio. About a year ago, a visit was paid to this nursery, where Mr. O'Brien immediately suggested the availability of one of the tropical fishes belonging to the family of cichlids. The animal indicated has long been familiar to aquarists and fanciers under the name of the jewel fish, technically known as Haemichromis bimaculata, native in Africa, from Egypt to the Congo. A pair of these animals was therefore obtained and a study of their adaptability to laboratory use was begun. As will presently appear, they have proved for a variety of reasons to be most excellent material. Indeed, so well do they lend themselves to the investigation of various problems that it is the intention of the writer to make them the subject of a series of papers dealing both with certain details of normal development and with the effects of various experimental treatments. Thus work on the action of altered physical and chemical environments, including an effort to analyze thoroughly the results of x-ray radiation upon ova and embryos, is now under way. The special object of the present paper is to describe the more salient characteristics in the development, and in particular to indicate certain features which have reference to the determination of the embryonic axis. A brief description of the general breeding habits and method of handling the adults and eggs will first be in order.

CHARACTER OF MATERIAL AND METHOD OF HANDLING

Haemichromis is a stagnant-water fish which does best at temperatures of from 23° to 26°C. Those used for breeding are approximately 7 to 8 cm. in length, and a single pair can be kept indefinitely in a balanced aquarium of 25 to 30 liters' capacity. Such a pair, when fed daily on fresh earthworms, or in winter on raw hamburg, will produce a batch of 500 or more eggs every three or four weeks practically continuously. Thus the writer has obtained from the pair acquired one year ago fifteen batches of eggs. The water used should have a pH of 6.5 or 7. Since most tap-water is more alkaline than this, it must be neutralized by HCl, or as the writer has found preferable, special water may be made up by adding to 1 liter of distilled water 0.263 gram of NaCl and 0.079 gram of $CaSO_4$. This when placed in an aquarium with plants furnishes an ideal medium.[1] When used pure without contact with older fish, earth, and plants, however, it is not entirely satisfactory for developing embryos. It is thought from results so far obtained that the embryonic defects produced by it can be overcome by the addition of a small amount of $MgSO_4$. As indicated, the whole subject of the effects of various ions on eggs and embryos is still under investigation, and will be considered in a later communication. As the water in the aquarium evaporates it should be replaced by more of the original water, not distilled water, since the plants and fish appear to utilize the excess salts. As regards the plants, it has been found advisable to place them in pots, and then to protect them by covering with cut-off lamp chimneys or some other device. Otherwise these fish, like most cichlids, will tear them to pieces. However, since under the conditions described a very rich growth of algae soon covers everything, a large stock of other plant material is unnecessary. For reasons connected with breeding the bottom should be covered with 5 to 6 cm. of sand, and somewhere on this should be placed a small clay flower pot, bottom up. For the latter it is sometimes convenient to substitute pieces of tile which can be laid upon a glass plate placed at a rather steep angle against the side of the aquarium.

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[1] For this formula I am indebted to Mr. J. T. Charleson, of the Tricker Nurseries.

Under these conditions the fish deposits her eggs on the crock or tile, from which they can be readily removed by a pipette of the proper size. Or, if desired, the entire pieces of tile with the eggs attached can be placed in finger-bowls for observation or treatment. When allowed to remain in the aquarium, the parent fish, like many of the cichlids, jealously guard the eggs, and take turns in fanning fresh water over them. This, however, is not necessary for their perfectly normal development, as is proved by the history of the numerous ova which have been removed. At the end of approximately forty-eight hours the parents start taking the eggs from the crock and carrying them in their mouths to a pocket which they have hollowed out in the sand. Here they seem to break the egg membrane while still in the mouth, and, as it were, shell out the young fish. After this task is completed, the parents continue to watch over the young for four or five days, at the end of which period they begin to swim about in a dense school, still guarded by the adults. Previous to this the young fish have usually been moved once or twice, being carried as before in the mouths of the parents. This habit, which is common among cichlids, has certain interesting variations under abnormal conditions, but it is not a subject germane to the present discussion. A further account of the breeding procedure of these fish, together with a brief and inaccurate description of some of the stages of development, is furnished by Von A. Konnertz ('13), and another even briefer account by F. Hoepfel ('13). The general habits and anatomy of the whole group of cichlids, with especial emphasis on the matter of oral gestation, is also discussed by J. Pellegrin ('04) in his "Étude des poissons de la famille Cichlidés." Turning now to the general subject of early development, with special reference to its bearing upon embryonic symmetry, we find the following situation.

GENERAL PROBLEM

The question as to what factors determine the embryonic axis in any ovum is always an important one, and has long been under investigation. Thus in many eggs it has been shown that this axis has some direct relationship to the shape of the ovum, the direction of the first cleavage plane, the entrance point of the sperm, or some combination of these factors. In the case of the eggs of teleost fishes, however, those so far investigated have yielded no clue as to what organization or processes are responsible for fixing the symmetry. Agassiz and Whitman ('84, '85, '89), it is true, believed that in the pelagic eggs of Ctenolabrus and probably other forms which they investigated, the first cleavage plane coincided with the longitudinal axis of the embryo. Morgan ('93), however, in his study of the eggs of Ctenolabrus and Serranus, was unable to discover any factor which fixed the embryonic symmetry. In the egg of the toadfish, Ryder once thought that light had some influence in this connection, and later that the position of the embryo was determined in some way before the egg left the body of the mother. Yet Clapp ('91) appears to have shown that no such relationship exists, and the significant factors in this case also remain unknown. Bataillon ('96) has claimed that in the perch and certain other teleost eggs the blastodisc is on the side. The first furrow, according to his account, is then vertical and the longitudinal axis of the embryo is always at right angles to this furrow. These statements appear not to have been controverted with respect to the special forms concerned, but they seem to lack confirmation, and in view of the other observations indicated, must remain in doubt for the present.

It is with respect to this problem that the egg of Haemichromis seems to present certain unique features which will be especially stressed in the present paper.

GENERAL CHARACTER AND EARLY DEVELOPMENT OF THE EGG

In the case of this animal the first feature to be emphasized is the fact that the egg is not spherical, like those of most teleosts known to the writer, but is distinctly ovoid.[2] Thus thirty eggs selected at random from two different batches show an average length of 1.25 mm. and a diameter in the broadest dimension of 1.01 mm.—a difference of 0.24 mm. The term 'broadest dimension' is used because these eggs are not symmetrical. This is due to a marked flattening on one side, such that the average diameter perpendicular to this flattening is only 0.86 mm. It is this flat side which under normal circumstances is pressed against and adheres to the substratum on which the egg is deposited (fig. 1). This relationship of the side of an ovoid teleost egg to a surface is, so far as the writer has been able to discover, unique, and as will presently appear, seems to be a condition of significance. Whether the flattening of this side is due to the pressure during deposition or is a preformed asymmetry which exists when the egg leaves the oviduct has not been demonstrated with absolute certainty. The writer, nevertheless, feels very sure, for several reasons, that the flattening is actually produced by the pressure at the time the egg is deposited. In the first place, it has been ascertained that the egg membrane is relatively soft as well as slightly sticky at this time. Hence eggs removed at this period are found to be very easily flattened or otherwise malformed by the contact of the pipette unless care is used. Furthermore, it occasionally happens that an egg will fail to be extruded by the female at the normal angle. In such cases, instead of the side of the egg adhering to the substratum, it will be attached near its end, which is then flattened instead of the side. Likewise, eggs are sometimes crowded against one another in deposition so that they adhere together, and when this occurs the areas of contact are flattened.

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[2] The egg of Osmerus mordax is described by Agassiz and Whitman as being oval. This condition, however, is very slightly emphasized, since the two dimensions of the ovum are given as 0.75 × 0.80 mm., a difference of only 0.05 mm. The eggs of certain Blennys and some others are also noted as ovoid or elliptical (Dean, '95).

The second significant feature regarding these ova is the fact that the blastodisc appears not on the side opposite the substratum, as in the spherical egg of the toadfish, but at one end. As might be expected from a knowledge of the situation in other teleost eggs, it is at this end also that the membrane is pierced by the micropyle, a structure which is always plainly visible under a low-power binocular. With respect to the formation of the blastodisc I am able to state that it exists within a few seconds after laying and previous to fertilization. It is, I believe, augmented slightly previous to cleavage, and possibly afterward as noted by Agassiz and Whitman. There is, however, no obvious streaming of protoplasm from other parts of the egg in its direction as is described, for example, by Wilson ('89) in Serranus and by Ryder ('82) in the cod. In so far as such a movement occurs, it is apparently very gradual and slight. As revealed by sections, the protoplasmic cap is at this time in continuity with a very delicate layer of superficial protoplasm, which entirely envelops the ovum, as well as with protoplasm as yet unseparated from the underlying yolk, so that, as in other teleost eggs, opportunity for additions from both these sources is present.

Because of attention to other problems, an extensive study of the details of fertilization has not been attempted. Nevertheless, some observations have been made, and so far as these have gone, they have revealed nothing unusual. The membrane about the egg is full of sperm which have adhered to it, but they are only able to enter through the micropyle. In the one instance most carefully observed, it was found that as the membrane receded from the ovum, a cone of protoplasm adhered to the tip of the micropyle and in this cone five sperm were noted. Four of them were almost within the egg and one farther away (fig. 2). The latter entered later, and eventually the cone separated from the micropyle. It is possible that other sperm entered before observation was begun. No polar bodies have so far been observed, although they have been searched for.

The first cleavage furrow follows fertilization in an hour to an hour and a half, depending on the temperature. The second takes place within twenty to thirty minutes, and, as usual, the ensuing divisions occur more rapidly. This process soon results in the typical cap of cells which is raised up, button-like, upon the yolk (fig. 3), while sections reveal a slight segmentation cavity within. As yet I have made no careful study of the relations of the parablast, but so far as observed they appear to be similar to those described in other teleosts; i.e., Wilson ('89) in Serranus, Agassiz and Whitman ('84, '85) in Ctenolabrus, and others. About ten hours after fertilization, this cap begins to flatten down upon the yolk and at the same time or a little later it becomes apparent that one side of the blastoderm is slightly nearer to the vegetal pole than the other (fig. 4). Observations upon fifty eggs removed from the crock and kept in separate watch-glasses until the position of the embryo was apparent indicate that with very few exceptions this low point marks the so-called dorsal lip of the blastopore, and hence the posterior end of the embryo. Indeed, out of the fifty there were but three exceptions to this rule, and in these cases the lack of coincidence seemed to be rather less than 90°. Though the dorsal lip is thus more active at the start, its advantage, so far as the amount of yolk which this portion of the blastoderm rim covers, is brief. Very shortly the remainder of the rim overtakes it, and from then on all parts advance at an equal rate toward the vegetal pole, which is reached in about twenty to twenty-two hours after fertilization at a temperature of 25° to 26°C. At the beginning of this epibolic process, the blastoderm as viewed from the surface appears equally opaque at all points (fig. 4). Presently, however, the side where the embryo is to form shows a broad area of greater opacity, particularly in fixed material (fig. 5). This condition also extends around the rim of the blastoderm, indicating the existence of the characteristic germ ring, as partially shown in drawing (*b*) of this figure. By the time the area of overgrowth has a little more than half covered the yolk, the outline of the embryo is faintly visible in the midst of the embryonic shield, and as the process is concluded embryo and shield are both distinctly marked out (figs. 6, 7, 8).

From these figures another feature will be apparent which is perhaps worthy of note. With respect to the relation of the embryonic anlage to the blastoderm, Haemichromis is similar to forms like Ctenolabrus, but differs notably from others, such as Serranus as described by Wilson ('89). From his account it would appear that at the time the blastopore closes the midpoint of the embryo about coincides with the original midpoint of the blastoderm; i.e., the middle of the animal pole, the anterior end of the embryo being almost as far to one side of this pole as the posterior end is to the other, as determined by the position of the oil globule. Indeed, Wilson states that: "In the spreading of the blastoderm the posterior pole or tail end of the embryo remains as a comparatively fixed point, while the anterior pole, *a. p.*, Fig. 35, travels rapidly in the direction of the arrow"—i.e., anteriorly. This certainly is not the situation in Haemichromis. In this animal the embryo never normally has its midbody region over the center of the animal pole. Instead, the embryonic anlage, as soon as it becomes visible, extends from near the center of the animal pole downward to the edge of the blastoporal lip, and as the latter advances over the yolk, the posterior end of the embryo keeps pace with it. The anterior end, on the other hand, remains virtually stationary. At least it never extends more than slightly onto the opposite side of the animal pole, as indicated both by the relation to the micropyle and the shape of the egg (figs. 7, 8). Thus the lengthening in this case is almost entirely in one direction up to the time of hatching.

The embryo being clearly visible at this stage, it is now possible to note certain interesting facts regarding the relation of its axis to the shape of the egg. It will be recalled that the situation in this case is unlike that existing in the toadfish in two significant respects. The egg of the latter, though like that of Haemichromis in being deposited on a solid substratum, is virtually spherical, and is placed in such a way that the blastoderm is opposite to the surface to which the egg is attached. Under these circumstances, according to Clapp, the embryo may then develop along any one of the infinite number of meridians extending from one pole to the other. In Haemichromis, on the other hand, the egg, as already indicated, is not spherical, and furthermore is not deposited with the animal pole opposite to the substratum. Instead, it is oval, and lies on its side, with the animal pole at one end. It has previously been stated that the embryo then develops along one side of the oval yolk in such a way that when completed its head lies at the original animal pole of the egg and its tail at the other in the region of the closing blastopore. There now remains to be added the significant fact that the half of the egg upon which the embryo develops is virtually always the half opposite the substratum. It is true that the embryonic axis does not always lie along the exact middle of this half, though it approximates this in a considerable percentage of cases. The situation will be rendered more definite by reference to data from a number of eggs removed from the crock and oriented flat side down in a watch-glass. The relationships of the embryo and eggs were recorded by making a diagram of each egg subsequent to its orientation. Great care was taken not to disturb the ova, and when the embryos appeared the position of each was recorded upon the corresponding diagram. It will be realized that if by chance an egg was disturbed, this fact would be apparent because of the change in the relations of its distinctive parts to the other eggs and to the bottom of the watch-glass. The results are shown in table 1.

In this and the following tables, except where otherwise stated, all eggs having the embryos within about 45° of either side of the middle line were counted as in the middle, and all those with the embryos roughly between 45° and 90° from the middle were counted as on the side. It will be noted from

TABLE 1

Eggs right side up

TABLE 2

Eggs wrong side up

Part A. Eggs oriented previous to cleavage

BATCH	EM	M	S	TR	EM	M	S	FS
I	2	4	9		19	20	24	
II					7	10	10	
III	3	5	8		5	5	4	
IV	5	7	19					
V	9	19	13					
VI	1	3	22	1	3	9	21	5
X	2	4	6		6	7	5	
XI					4	9	16	
Totals without VI		39 41.48%	55 58.52%			51 46.33%	59 53.47%	
Totals with VI	22 18.48%	42 35.29%	77 64.71%	1	44 30.34%	60 41.38%	80 55.17%	5 3.45%
		Total eggs, 119				Total eggs, 145		

Part B. Eggs oriented in medium or late cleavage

BATCH	EM	M	S		EM	M	S	
I	3	7	11		10	14	5	
VII	1	10	8		4	5	13	
VIII	4	14	20					
IX	28	49	18					
XI	14	33	19					
Totals	50 26.45%	113 59.79%	76 40.21%		14 35.94%	19 51.36%	18 48.64%	
		Total eggs, 189				Total eggs, 37		

Part C. Eggs oriented in four-cell stage

BATCH	EM	M	S
VIIIa	5	13	13
VIII	11	20	15
X	4	7	8
Totals	20 26.31%	40 52.63%	36 47.37%
	Total eggs, 76		

	EM	M	S		EM	M	S	FS
Grand totals	92 23.95%	195 50.78%	189 49.22%		54 31.86%	79 43.40%	98 53.84%	5 2.76%
		Total eggs, 384				Total eggs, 182		

EM = embryo in exact middle. M = embryo in middle, and includes the eggs under EM. S = embryo on the side. TR = embryo transverse. FS = embryo on the flat side. Batch VI, part A, was removed from the crock thirty minutes after laying, but was not actually oriented until the two-cell stage. Batch XI, part B, was left on the crock throughout the experiment. Batch VIIIa, part C, was removed about thirty minutes before cleavage, but was not oriented until the four-cell stage. Batches IV, V, VI, VII, VIII, and IX are from the same layings as batches I, II, V, VI, VII, and VIII, respectively, in table 5, and hence constitute controls for these batches in the latter table.

the summary of parts A, B, and C of the table that out of 384 eggs from eleven different batches so oriented, 50.78 per cent are in the group classed as having the embryos along the middle of the longitudinal axis of the ovum. In the remaining 49.22 per cent the embryos, while classed as on the side, were, as indicated, never more than 90° from the median axis; i.e., they were never on the flat side. Furthermore, it will be observed that of the 50.78 per cent counted as in the middle, 47.12 per cent of these, or 23.95 per cent of the total, were in the exact middle as nearly as this could be determined. The specimens here recorded were rather exactly studied. Many others, some of which are not included in any table, were observed sufficiently to make certain that the embryos at least were not on the flat side. In fact, excluding the cases considered later under the effects of centrifuging, it may be stated that out of 2598 eggs only seventeen embryos have ever been discovered squarely on the flat side of the ovum. Six of these had been subjected to some abnormal treatment to be described below, while three of the others may owe their abnormality to handling. Thus, in spite of obvious variations, it is believed that these figures together with certain other data are enough to indicate a very decided tendency toward median placement. Nevertheless, at least eight of the above seventeen exceptions were cases in which the eggs were never even removed from the substratum. That this small percentage (0.52 per cent in cases where no disturbing influence could be assigned) does occur would then indicate either that the normal relation to the substratum is not always effective in influencing the position of the embryo, even when operative from the moment of laying, or else that some other unknown factors are involved. Such a factor, of course, may well be an organization of the egg which exists previous to laying, and which is usually, but not inevitably, related to the position in which the egg is deposited.

An attempt to solve this problem made immediately necessary a thorough study of the following questions: 1) To what extent is the position of the embryo affected simply by the relation of the egg to the substratum; 2) to what extent is it the result of the action of gravity; and, 3) what, if any, relation has its position to the first cleavage plane? Observations designed to answer these questions were undertaken with the following results.

RELATION OF THE EMBRYO TO THE SUBSTRATUM

In order to determine the effect of the substratum, eggs were removed from the crock and oriented flat side up in watch-glasses. Contrary to expectation, the embryos in almost every instance continued to develop along the side of the ovum which would have been away from the substratum had the eggs remained undisturbed. Thus figures 9 a and b illustrate the cases of two ova of the same batch removed at the same time during late cleavage, one (a) being oriented flat side up, and the other (b) in the normal position with the flat side against the substratum. It will be observed that in both cases the embryo bears the same relation to the flat side of the egg; i.e., it is squarely in the middle of the opposite or normal side. It is perhaps further significant that, even though the egg, oriented upside down, has developed a slight secondary flattening upon the side now against the substratum, this has not prevented the development of the embryo on this side. A more adequate conception of the situation is gained from an analysis of the results obtained from all the eggs which were removed from the crock at various stages and oriented wrong side up (table 2) as compared with the results from eggs normally oriented, to some of which attention has already been called in table 1. It will be noted that both of these tables are divided into parts. Part A in each contains data from eggs removed from the crock previous to cleavage, while part B shows the results from eggs removed during medium or late cleavage.[3] Part C in table 1 gives the record of a few ova which were in early cleavage, and has no counterpart in table 2. The significance of these divisions will appear presently.

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[3] Batch XI of part B was, as indicated, left on a piece of tile until hatching, the piece being removed from the aquarium to another dish previous to cleavage. Since these eggs were never disturbed with relation to the substratum, they afford a particularly good example of the type of egg included in part B, though their treatment differed from that of the others.

Considering first the percentages derived by grouping together eggs removed at all stages, it appears that whereas out of 384 eggs oriented right side up, 50.78 per cent were approximately in the middle and 49.22 per cent on the side, in the case of 182 oriented wrong side up, 43.4 per cent were approximately in the middle and 53.84 per cent on the side. From this one might at first conclude that inversion of the ovum has a slight tendency to displace the embryo from the median line. While this may yet prove to be true, the figures so far obtained are not sufficiently consistent to prove it, as a more complete study of the results will indicate. Before pursuing this topic to its conclusion, however, it will be convenient to point out first certain other facts which the tables reveal.

It will be seen that parts A in both tables 1 and 2 show a higher percentage of laterally placed embryos (58.52 per cent and 53.47 per cent, respectively), while in parts B the situation is reversed, with 59.79 per cent median placements in table 1 and 51.36 per cent in table 2. Part C of table 1 contains data from eggs removed later than A but earlier than B, and the percentage of median embryos (52.63 per cent) is likewise between that in part A and part B. From this one might reasonably conclude that the earlier an egg is disturbed, the greater is the likelihood of upsetting the orientation of the embryo. This, furthermore, is borne out by certain other meager but suggestive data. Thus it happened that all the eggs in batch VI of parts A in both tables were removed from the crock quite early, and left lying in various positions for about half an hour. They were then disturbed again for the purpose of orienting, and it will be noted that both normally placed and inverted ova of this group show an excessive number of embryos on the side. Not only this, but those inverted show five cases in which the embryo was actually on the side opposite the normal. This of course is only 2.76 per cent of the total, but since it has so rarely if ever been observed in many hundred normally oriented eggs, it has an appearance of significance. Again the above conclusion receives slightly more confirmation from a single egg of a recent batch which was removed to a watch-glass five seconds after laying. This is by far the earliest removal on record, and it is therefore of interest that the embryo in this case developed across the side opposite to normal. Unfortunately, the orientation of this egg during development was not recorded.[4]

Returning now to the effect of inverting the eggs, it might be supposed that such treatment would be a more serious form of disturbance than merely removing them from the crock. If such were the case, however, a higher percentage of the eggs in table 2, part A, should be laterally placed than in table 1, part A. Yet exactly the contrary is true. In part B, the expected relationship of a lower percentage of approximately median embryos among the inverted eggs than among those normally oriented does occur, thus accounting for the general percentages presented at the beginning of this discussion. If earlier disturbance including inversion were more effective, however, the result should be less apparent in these eggs in part B than in those in part A.

We are, therefore, driven to this general conclusion with respect to the relation of the substratum to the placement of the embryo. Although there is evidence that a general disturbance of the eggs at an early stage may have some tendency to upset the normal orientation of the embryo, there is not sufficient evidence at present to indicate that the specific disturbance produced by a changed relation of the egg to the substratum has any particular effect. Hence, the position of the embryo is either due to other factors or else the effect of the substratum is produced at an earlier stage than these experiments involve. A readily conceivable method by which this might occur suggests itself, and demands consideration before passing to the next topic.

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[4] This is one of the six eggs referred to above as having abnormally placed embryos. The other five are those just indicated as belonging to batch VI and oriented upside down.

From what has already been said, it is clear that there are no markings on the egg itself which distinguish one side from another. Hence, so far as the preceding experiments are concerned, it might be said that while the substratum is not directly responsible, the flattening which it produces in the membrane and secondarily in the egg may be so. This, of course, would immediately explain the tendency for the embryo to develop opposite this side, no matter how the egg is turned. It must be admitted at once that the flattening may well be the agent which at a very early stage affects the organization of the egg in a way to control at least partially the orientation of the embryo—a hypothesis which is now being investigated. That this flattening is invariably effective, however, is clearly disproved, not only by the five eggs already cited, in which the embryos developed squarely upon the flattened side, but by other similar evidence presented in the remainder of the paper.

RELATION OF THE EMBRYO TO GRAVITY

As regards the second problem, i.e., the effect of gravity, the following situation exists: In the case of one batch of eggs the crock, instead of being placed upside down, was laid on its side. The eggs were then deposited within it, partly upon one of its lateral sides, and partly upon the side in contact with the bottom of the aquarium. Under these circumstances the fish during the act of deposition on the lateral side moved some of the time approximately horizontally and some of the time vertically. Hence the ova in this location were placed with their longitudinal axes horizontal, vertical, or somewhere in between. In the case of those horizontal, it is then obvious that the line of the perpendicular would pass through the sides of the egg, though the latter had a perfectly normal relation to the substratum. Those ova deposited on the lower side of the crock, of course, all lay flat side down with their longitudinal axes horizontal. After removing a few eggs in the earlier stages for other purposes, the remainder were allowed to develop until the

the embryonic axes were apparent. The entire crock was then removed, broken into a couple of convenient pieces, and immersed in preserving fluid with the eggs undisturbed. At the same time the pieces were marked in such a way that those eggs on approximately the lower side were roughly separated from those on the lateral side. It is evident, of course, that such a division could not be exact, since the surface curved, and there was no line practically determinable which would precisely separate the lateral side from the lower. Nevertheless, there was a considerable region in each location whose position was indubitable. Subsequently a careful record was made of the positions of all the ova, together with the respective positions of their embryonic axes. The results are shown in table 3.

TABLE 3

Eggs on side of crock					*Eggs on bottom of crock*	
Egg vertical		*Egg horizontal*				
M	S	US	M	LS	M	S
First count						
111	73	55	44	3	36	26
60.33%	39.67%	53.93%	43.13%	2.94%	58.07%	41.93%
Second count						
107	64	63	40	3	36	25
62.58%	37.42%	59.44%	37.73%	2.83%	59.02%	40.98%
Average						
61.40%	38.60%	56.73%	40.38%	2.89%	58.54%	41.46%
Part A. Cases where eggs were very clearly on the side						
13	10	31	9	2		
56.52%	43.47%	71.42%	21.43%	7.15%		

M = embryo in the middle of the egg. S = embryo on the side of the egg. US = embryo on the upper side of the egg. LS = embryo on the lower side of the egg. All eggs on the bottom side of the crock were, of course, horizontal, but with their flat sides also horizontal. Those on the lateral side, on the other hand, while horizontal, had their flat sides vertical. Note how the percentages correspond between the eggs placed vertically on the side of the crock and those placed horizontally on its bottom.

It will be noted that the position of the ova on the lateral side are all grouped under two headings; i.e., either vertical or horizontal, whereas some, as indicated, were of course not exactly either. Such a division seemed necessary, inasmuch as it was, of course, impractical to list all the possible infinite variations. Hence the tabulation was accomplished by arbitrarily placing all eggs approximately 45° to either side of the vertical, in the vertical column, and all outside of that group, in the horizontal column. Even so, there were obviously some which would be on the border line. In order, therefore, to ascertain whether the writer's judgment on such cases would be reasonably consistent, the eggs were classified at two different times several hours apart. The two sets of results are tabulated as well as their average, and it will be noted that the sets are decidedly consistent, the differences in each tabulation being in the same direction and only slightly different in amount.

The conclusions to be drawn from these figures seem obvious. If an egg is either in a vertical position with respect to its longitudinal axis, or is lying horizontally with the flat side down (i.e., on the lower side or 'bottom' of the crock), the embryo has a somewhat greater tendency to lie along the median line of the ovum than to either side of it.[5] On the other hand, when the egg is attached to a vertical surface, but with its long axis horizontal, there is a decided tendency for the embryo to appear on the upper side. It is less likely to be in the middle and almost never on the lower side. To this extent, then, gravity seems to have some influence. This fact is further emphasized by the following: In the second tabulation forty-two eggs were selected which were attached near the upper edge of the piece of crock which had constituted the lateral side. These ova were, therefore, unequivocally attached to a vertical surface as compared with some lower down, whose position was not so evident. As would be expected if the above interpretation be correct, the percentages from this selected group, when considered separately, show the conditions indicated even more clearly (table 3, A).

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[5] The extent to which the percentages of embryos with median placements in these two groups coincide with the percentage of median placements among the eggs removed and oriented right side up, as shown in part B of table 1, is noteworthy. Such coincidence is, of course, to be expected according to the hypotheses advanced in this paper.

To test this conclusion yet further, fifty-seven ova from recent layings (mostly included in table 4, D) were removed from the crock about thirty minutes before cleavage began, and oriented on edge, i.e., with the flat sides facing laterally. From this point on, they were then in a position exactly corresponding to eggs attached to a perpendicular surface with their axes horizontal, with the additional circumstance that the ova now under consideration had a new area of pressure on the side now against the watch-glass. Of these fifty-seven eggs, nineteen had the embryos exactly along the middle of the side opposite the original lower and flattened surface, i.e., the normal side. Nineteen had the embryos exactly on the present upper side, about 90° from normal, eleven others were slightly displaced toward this side, while the remaining nine were decidedly on the present lower side.

As a final test of this tendency of embryos in horizontally placed eggs to appear on the upper side, and also in an effort to determine at what approximate stage the influence of gravity was most effective, two further experiments were performed, again with the eggs attached to their substrata. In the first place a piece of tile with seventy-eight eggs upon it was left in the aquarium at a very slight angle with the vertical. The condition of these eggs was thus essentially the same as those deposited on the lateral side of the crock. When examined at fifty-five hours, out of sixty-three eggs which had been horizontal with respect to their long axes, and with their flat sides practically vertical, eighteen showed the embryos to one side of the median plane of the egg, and of these fifteen were toward the upper side. In the second experiment a piece of tile with sixty-three eggs was removed previous to cleavage and placed horizontally in a finger-bowl. At thirty-one hours it was examined, and the positions of all embryos recorded. All were normal, i.e., either along the middle of the egg opposite the flattening or slightly to either side. The tile was then placed in a vertical position and examined at the end of twenty-four hours. Out of twenty-four eggs which were now horizontal with respect to their long axes and with their flat sides vertical (the same as eggs oriented on edge), only two embryos had shifted toward the upper side, while two others seemed to have shifted toward the lower. Also out of thirty-nine which had their long axes vertical, or within 45° of it, three seemed to have shifted. Hence none of these shifts was probably significant, and it seems reasonable to conclude that gravity, in order to be effective in causing the side of the egg which is to contain the embryo to turn upward, must act quite early. It remains to add concerning these two groups that in the first group there were fifteen eggs whose long axes were vertical or within 45° of it, and among these eggs ten had the embryos almost exactly opposite the flattened side. In the second group in which all the eggs were horizontal, but with the flat sides ventral, for the first thirty-one hours, forty-one out of the sixty-three embryos lay exactly opposite the flattened side. This serves again to emphasize the general principle that the embryos tend to appear opposite the flattening. It also suggests that this tendency is augmented when the sagittal plane of the egg and embryo is perpendicular, or nearly so, owing presumably to the effect of gravity previously demonstrated.

At present, then, it may be concluded that, in so far as gravity is effective, it tends in eggs properly oriented to make the sagittal embryonic plane coincide with the vertical, and also to place the embryo on the upper side of the ovum. However, that the effect of gravity, to the extent that it occurs, is due to some action upon the organization of the egg itself is, of course, by no means demonstrated. In view of previous and subsequent evidence, it appears more likely that the organization is already fixed by the initial pressure of the flattened membrane or otherwise. It then happens that the side destined to contain the embryo is lighter than the other, and the egg being partially free to turn within its membrane, this side will tend to place itself uppermost. Thus, as would be expected, ova in which the long axes are vertical are not affected. Nor, as already shown, are ova which have been completely inverted with the flat side horizontal but uppermost. The reason for the latter fact is probably because the embryo in such cases lies in the vertical plane even though upside down, and hence is in relative equilibrium compared with one halfway toward the upper side. In any event, it would seem that to be effective the gravitational factor must have opportunity to act very soon after laying, and that under normal conditions it is probably of rather minor importance.

RELATION BETWEEN THE EMBRYONIC AXIS AND THE FIRST CLEAVAGE PLANE

Coming now to the third question propounded above: What, if any, relation is there between the embryonic axis and the first cleavage plane? As already stated, there have been conflicting views regarding such a relationship in other teleosts, with all the more recent evidence opposed to its existence. In the case of an ovum having the rather definite symmetrical peculiarities of Haemichromis, therefore, the answer to this problem seemed of peculiar interest.

It has been shown in the preceding sections of this paper that the axis of the embryo has a fairly definite connection with the flattened side of the egg, which normally adheres to the substratum. Also, under certain circumstances, there is some evidence that gravity may play a somewhat secondary rôle in the placement of the embryo. The questions now to be answered, therefore, are with respect to the relationships which may exist between the first cleavage plane and these other factors; i.e., gravity, the flat side of the ovum, and the embryonic axis. The solution of these problems was sought through four more or less separate experiments, the results of which are summarized in table 4 and explained below.

Part A of this table shows what happened in the case of eggs from four different batches which were removed from the crock previous to cleavage, and oriented right side up in watch-glasses. Part B is a similar record of eggs which

TABLE 4

Part A. Eggs oriented right side up

BATCH	CLEAVAGE PLANE VERTICAL			CLEAVAGE PLANE HORIZONTAL			CLEAVAGE PLANE INTERMEDIATE		
	E and CP coincide	E and CP do not coincide		E and CP coincide	E and CP do not coincide		E and CP coincide	E and CP do not coincide	
		E ⊥ to CP	E I to CP		E ⊥ to CP	E I to CP		E ⊥ to CP	E I to CP
I	2	3	3	1				3	1
II	2	3	2	1			1	2	
III	5	0	2			2	2	0	1
IV	1	2	2		1		4	1	
Total	10	8	9	2	1	2	7	6	2

Part B. Eggs oriented right side up but attached to tile

BATCH	CLEAVAGE PLANE VERTICAL			CLEAVAGE PLANE HORIZONTAL			CLEAVAGE PLANE INTERMEDIATE		
	E and CP coincide	E and CP do not coincide		E and CP coincide	E and CP do not coincide		E and CP coincide	E and CP do not coincide	
		E ⊥ to CP	E I to CP		E ⊥ to CP	E I to CP		E ⊥ to CP	E I to CP
I	6	4	6	3	5	5	4	8	11
Total A B	16	12	15	5	6	7	11	14	13

Part C. Eggs oriented wrong side up

BATCH	CLEAVAGE PLANE VERTICAL			CLEAVAGE PLANE HORIZONTAL			CLEAVAGE PLANE INTERMEDIATE		
	E and CP coincide	E and CP do not coincide		E and CP coincide	E and CP do not coincide		E and CP coincide	E and CP do not coincide	
		E ⊥ to CP	E I to CP		E ⊥ to CP	E I to CP		E ⊥ to CP	E I to CP
I	12	7	5		7	4		4	6
II	2		1	3	1				6
III	3	5	4		1	2	2	5	3
Total	17	12	10	3	9	6	2	9	15

Part D. Eggs oriented on edge

BATCH	CLEAVAGE PLANE VERTICAL			CLEAVAGE PLANE HORIZONTAL			CLEAVAGE PLANE INTERMEDIATE		
	E and CP coincide	E and CP do not coincide		E and CP coincide	E and CP do not coincide		E and CP coincide	E and CP do not coincide	
		E ⊥ to CP	E I to CP		E ⊥ to CP	E I to CP		E ⊥ to CP	E I to CP
I	2	6	2	4	3		3	1	2
II	1		2			1	5		2
III	1	3	1		4	1	2	1	1
Total	4	9	5	4	7	2	10	2	5

Summary of results in percentages

PARTS	A	B	A AND B	C	D
Cleavage plane vertical	57.44	30.77	43.43	46.99	37.50
Cleavage plane horizontal	10.64	25.00	18.18	21.68	27.08
Cleavage plane intermediate	31.92	44.23	38.39	31.33	35.42
Embryo coincides with cleavage plane	40.43	25.00	32.32	26.50	37.50
Embryo perpendicular to cleavage plane	31.91	32.70	32.32	36.15	37.50
Embryo intermediate to cleavage plane	27.66	42.30	35.36	37.35	25.00

E = embryo. CP = cleavage plane. I = intermediate. ⊥ = Perpendicular.

had been laid on a flat piece of tile instead of crock and left there throughout observation. This tile was removed from the aquarium as soon as the eggs had been deposited, and was placed in a finger-bowl so that the flat side on which the eggs occurred was horizontal. The removal was, of course, accomplished previous to cleavage, and it is to be noted that the position of the removed tile in this case placed the eggs upon it in the same relation to gravity as were those removed separately and oriented right side up in watch-glasses. For this reason it seemed permissible to present a summary of the data from parts A and B combined as well as separately. Part C represents the data from three different batches of eggs, removed from the crock previous to cleavage and oriented upside down in watch-glasses. Part D shows the results in the case of eggs removed in the same manner and oriented on their sides. In each of these experiments the data was obtained by the same general method as in previous observations; i.e., after orienting, a diagram of each egg was made, and the direction of the first cleavage plane was marked upon it. The usual care was used not to disturb the eggs, and when the outline of an embryo was apparent, this also was recorded on the respective diagram. The results can best be analyzed by considering the various factors under two main headings: 1) the relation of the cleavage plane to gravity and to the flat side of the ovum; 2) the relation of the first cleavage plane to the longitudinal axis of the embryo.

First, let us consider the relation of the cleavage plane to gravity. This can readily be done because of the fact suggested above that orientation of these ova with respect to gravity was known in every case. Thus, in all the experiments the substratum was horizontal, and in parts A, B, and C the flat side of each egg was parallel with it; i.e., in A and B, in contact with it, and in C, opposite to it. In D, of course, the flattened sides of the ova were perpendicular.

From the data contained in parts A and C of this table, it might then be concluded that there is a slight tendency for the cleavage plane to be perpendicular. This would follow from the fact that in A, for example, the percentage in the perpendicular group is fairly high, that in the intermediate group next, and that in the horizontal group quite low. It will be noted, however, that in part C this tendency is much less marked, while in part B it is hardly apparent at all. Furthermore, because of circumstances already referred to, it is obvious that in those cases just considered the cleavage plane is perpendicular not only with respect to gravity, but also to the flat side of the egg. Thus the question arises: Is it the relation to gravity or to the flat side which is significant? This query is an important one, and it is easy to devise a test which will answer it. This has been done in the data presented in part D, where the eggs were oriented on edge with the flat side facing laterally. So far as these data now go, they show a small excess of vertical cleavages, 37.50 per cent, as against 27.08 per cent horizontal and 35.42 per cent intermediate—thus seeming to indicate that the flat side in itself is not a very dominant influence. Nevertheless, it must be pointed out that the percentage of cleavage planes parallel to the substratum—i.e., horizontal, and hence in this case perpendicular to the flat side—show a distinct increase in this group over the horizontal percentages in the other groups. This increase in the horizontal cleavages in the eggs oriented on edge appears to lend support to the view that there is some tendency for the cleavage to be perpendicular to the flat side. Taking all the data together, then, it seems permissible to suggest that both gravity and the shape of the egg may each exert an influence which, in the case of eggs oriented with the flat side horizontal, will be in the same direction; i.e., such as to cause the cleavage to be vertical. Obviously, however, even these combined influences are only indifferently effective.

Secondly, as regards the relation of the cleavage plane and the sagittal plane of the embryo, it appears that the figures to a considerable extent confirm the conclusions of the more recent workers on other teleosts; i.e., the relationship, if any, between these planes is slight. Thus in groups A and C the percentage of coincidence between the cleavage planes and the sagittal planes of the embryos is respectively greater than, and equal to, the percentages where these planes are perpendicular to one another or intermediate. In B, on the other hand, the percentage of coincidence is decidedly lower than either of the others. On the face of the data, therefore, one might include that there is no relationship at all between the cleavage plane and that of the embryo. There are, however, certain other factors to be considered. It is a comparatively simple matter to say when the cleavage plane and that of the embryo coincide or are at right angles. When they are in neither of these classes, however, it is quite impractical to measure the exact angle by which these planes are separated. Hence such cases simply fall into the class of intermediate. Now, although it is difficult or impossible to express the exact relationship here in figures, it is nevertheless the distinct impression of the writer that in many of these cases classed as intermediate the embryonic plane comes nearer to coinciding with the cleavage plane than to being at right angles to it. It is hoped that continued observation may make possible a more precise statement in this regard, but at present it seems more accurate to say, not that there is positively no relationship between cleavage plane and embryonic plane, but rather that there may be a tendency toward coincidence. It is, however, a tendency only, and is very frequently partially or completely frustrated.

There remains to be discussed, in connection with the relation of cleavage plane and embryo, what might appear as a discrepancy between the data given in earlier tables with reference to the relation of the embryo and the flat side of the egg, and the data in the present table. Thus it was previously indicated that 50.78 per cent of the embryos were along the middle of the surface opposite to that flattened. If this be so, and a considerable per cent (e.g., 43.43) of the cleavages are perpendicular to this side in normally oriented eggs, why is the percentage of coincidence not greater in such eggs between embryonic axes and cleavage planes? To this the following may be said: The 50.78 per cent was obtained by classing embryos in the middle if they came within 45° of either side of the middle line. For the purpose of that study this was justifiable, but in the present instance the classification of what was vertical, both with respect to the cleavage plane and the plane of the embryonic axis, needed to be, and was, much more critical. Thus, if the per cent of embryos classed as exactly in the midline in tables 1, A, and 2, A (23.95 per cent and 31.86 per cent, respectively), is compared with the percentage of embryos exactly coinciding with vertical cleavage planes in the present study (37.20 per cent and 43.58 per cent in groups A, B combined, and C, respectively), the discrepancy is not so large, and what difference there is shows a greater degree of coincidence than might be expected, rather than less.

With respect, then, to the relation of the cleavage plane to gravity, to the flat side of the egg, and to the embryo we may conclude as follows: There is a slight tendency for the cleavage plane to be vertical, and also under circumstances where the two influences do not conflict, perpendicular to the flat side of the ovum. Partly in correlation with these facts there may also be a slight tendency for the sagittal plane of the embryo to coincide with that of the first cleavage, especially when the long axis of the egg is vertical, or when its flat side is horizontal and ventral.

EXPERIMENTS IN CENTRIFUGING

As a result of the foregoing observations on eggs with various orientations, it seemed reasonable to conclude that in the egg of Haemichromis there exists shortly after laying an organization of some sort which more or less definitely determines the region in which the embryo is to develop. Such being the case, it then became a matter of interest to discover to what extent this organization could be altered or its location changed by such a procedure as centrifuging. Particularly was this true in view of the fact that, although this method of attempting to alter the organization of various holoblastic eggs has been frequently used with interesting results, it has not, so far as the writer has been able to discover, been applied to a highly telolecithal egg such as that of the fish. Certainly it has not been applied to a fish egg with the characteristics of shape and organization possessed by this one.

The experiments performed under this heading may be divided into two main groups, 1) those in which the centrifuging took place previous to cleavage, and, 2) those in which it occurred afterward, but previous to gastrulation. In all cases the speed was approximately 1100 revolutions per minute, the eggs being in tubes whose ends were about 15 cm. from the center of the machine. The length of the treatment varied from thirty seconds to two minutes, as indicated in table 5, to which attention is now called.

In this table it will be noted that the two main groups of data indicated in parts A and B have each been subdivided as follows: First are those cases in which centrifuging produced no apparent effect so far as the position of the blastodisc and embryo was concerned; second are those in which the blastodisc was removed to an abnormal position, yet the embryo developed normally; third are the cases where the blastodisc was moved, and the embryo developed in an abnormal position; and fourth are a few instances where the blastodisc occupied an apparently normal position, but the embryo did not. In those groups where the blastodisc was displaced a further classification has been made (columns II and IV), indicating whether the embryo when it developed was on the side which had been occupied by the blastodisc (E S B), opposite to it (E O B), or intermediate (I). The reason for this will appear later. In these instances, therefore, the figures in columns II and IV represent the same eggs as those in columns III and V, respectively. In all cases where the embryos were normally placed they have further been classified as being in the middle of the normal side (M) or lateral to it (S). In columns V and VI showing the abnormally placed embryos, an attempt has been made to show

TABLE 5

Part A. Eggs centrifuged before cleavage

BATCH	TIME CENTRIFUGED, IN SECONDS	DEAD EGGS	I BLASTODERM NORMAL; EMBRYO NORMAL		II BLASTODERM ABNORMAL			III EMBRYO NORMAL		IV BLASTODERM ABNORMAL			V EMBRYO ABNORMAL			VI BLASTODERM NORMAL; EMBRYO ABNORMAL		
			M	S	E S B	I	E O B	M	S	E S B	I	E O B	FS	X	WE	FS	X	WE
I	30	3	5	8	2	2	2	1	5	1	0	1	0	1	1	0	0	0
	60	4	6	10	2	6	0	6	2	1	0	1	1	0	1	0	0	0
II	60	3	9	6	2	4	4	4	6	0	0	3	0	3	0	0	0	0
	120	4	5	8	0	3	1	1	3	0	0	1	0	1	0	0	0	0
III	60	5	12		1	0	1	0	2	1	0	0	0	0	1	0	0	0
IV	?	6	3	17	0	10	8	2	16	0	4	0	4	0	0	2	0	0
V	?	13	6	18	0	8	10	4	14	1	0	1	2	0	0	0	0	0
Total without III		33	34	67	6	33	25	18	46	3	4	7	7	5	2	2	0	0
Percentages		15.42	33.66	66.34	9.44	51.56	39.06	28.12	71.88	21.43	28.57	50.00	50.00	35.71	14.29	100		
Total with III		38	113		7	33	26	18	48	4	4	7	7	5	3	2		
Percentages		16.24			10.61	50.00	39.39	27.27	72.73	26.67	26.67	46.66	46.67	33.33	20.00	100		

Total eggs in part A, exclusive of twelve in batch III, 184. Percentage of M, 28.48; of S, 71.52.

Part B. Eggs centrifuged during cleavage

IV	?	7	2	8	4	4	6	8	6	1	0	0	1	0	0	0	0	0
VI	120	0	3	7	1	0	2	0	3	0	0	0	0	0	0	1	0	0
VII	90	0	9	11	0	2	0	2	0	1	0	0	0	0	1	0	0	0
VIII	90	22	7	14	0	1	0	0	1	1	1	0	1	0	1	4	0	0
Total without IV		22	19	32	1	3	2	2	4	2	1	0	1	0	2	5	0	0
Percentages		25.28	37.25	62.75	16.67	50.00	33.33	33.34	66.66	66.66	33.34		33.34		66.66	100		
Total with IV		29	21	40	5	7	8	10	10	3	1	0	2	0	2	5	0	0
Percentages		24.37	34.42	65.58	25.00	35.00	40.00	50.00	50.00	75.00	25.00		50.00		50.00	100		
Percentages of A and B		18.98	33.95	66.05	13.95	46.51	39.54	32.56	67.44	36.84	26.32	36.84	47.36	26.32	26.32	100		

Total eggs in part B, 90. Percentage of M, 34.44; of S, 65.56. Grand total of all eggs used, exclusive of twelve in batch III, 274. Percentage of M, 30.29; of S, 69.71.

E O B = embryo opposite blastoderm. E S B = embryo on side of blastoderm. FS = embryo on flat side of egg. M = embryo in middle of egg. S = embryo on side of egg. WE = embryo with head at wrong end of egg. X = other abnormal placements, including three cases in which the embryo was across one end of the egg, and two cases in the first series of batch II, in which the embryo was transverse.

The eggs in batch III were stained as noted in the text, and in the case of the normal embryos, the numbers in the middle (M) and on the side (S) were not recorded.

Eggs in part B, batch IV, were centrifuged in the four-cell stage, the others in this part of the table all being in late cleavage.

Eggs in batch V, though removed from the crock thirty minutes from the beginning of laying, were left unoriented for about thirty minutes more before centrifuging; i.e., until just previous to cleavage.

Batches III and IV had no controls from these same batches.

how many were on the flat side of the egg (F S); with the anterior end at the wrong end of the ovum (W E); or in some other position requiring special description (X). No controls are shown in this table. As a matter of fact, however, the eggs indicated in tables 1 and 2 constitute the controls, since they were all from the same pair of fish, and with the exception of batches III and IV, table 5, they were from the same layings.

The first thing to be noted regarding these centrifuged ova is the fact that out of a total of 274, only 30.29 per cent were approximately in the middle, as against 50.78 per cent out of a total of 384 normally oriented, untreated eggs. It thus appears immediately that centrifuging tends to a considerable extent to upset the usual orientation of the embryo. A further examination of the table then shows that while a large part of this disturbance has resulted only in an increased number of embryos lying to either side of the middle line, there are also quite a number, 9.09 per cent of the 286 centrifuged, which are more radically displaced. This is not a large percentage, but when the number of these abnormal placements is compared with the number observed among the many hundred untreated eggs it is obviously significant. Thus, as already stated, out of a total of 2598 ova only seventeen (0.65 per cent) were displaced to the flat side, five of these were from eggs oriented upside down at an early stage, while one of the remaining four had been removed from the crock five seconds after laying and rather roughly handled. Three other eggs out of 875 carefully checked for exceptional features were also abnormally placed, one having developed around the side opposite the micropyle, another at right angles to the longitudinal axis of the ovum (this egg was unusual in being almost spherical), and the third with the head at the end away from the micropyle.

As contrasted with this, the table reveals the following conditions among the 9.09 per cent of abnormalities from the ova subjected to centrifuging. Of the twenty-six eggs within this group, sixteen are cases in which the embryo is on the wrong (flat) side of the egg, five are cases in which the head was at the end opposite the micropyle (fig. 10), three have the embryos developed across the pole, and two show embryos placed transversely with respect to the egg's longitudinal axis. Thus a fuller analysis only more completely confirms the previous conclusion that centrifuging may, and frequently does, change the normal relation of the embryo to the egg membrane.

With this conclusion granted, the question arises as to how the change is produced, in answer to which two main hypotheses at once suggest themselves. Either the entire egg is simply turned inside the membrane, or else certain parts of the egg, presumably the blastodisc and the yolk, alter their positions relative to each other and incidentally to the membrane. A further discussion of these alternatives will be reserved until certain further facts connected with centrifuging have been considered.

First, with respect to the relation of the blastodisc and the yolk it is to be noted that in the normal eggs of this fish the latter is fairly homogeneous, with small fat droplets scattered through it at rather regular intervals. During centrifuging, however, these droplets are made to coalesce, and the larger globules more or less concentrated upon one side of the ovum (fig. 10). After a period of twenty-four hours or more, these globules rise to the upper surface, regardless of their original position. This would naturally be expected, granting that they consist of lighter material such as fat, and are free to move. The latter condition, however, apparently did not exist previous to centrifuging. These movements of the droplets always occur, but may or may not be accompanied by movement of the blastodisc with respect to its normal relation to the micropyle. When such a shifting of the blastodisc does occur, however, it always tends to be toward the same side of the egg as that occupied by the droplets after their initial displacement, thus indicating a correspondence in specific gravity. This tendency, however, frequently fails to result in entire coincidence, apparently because, whereas the droplets are rather easily moved, the blastodisc, or possibly the entire egg including the blastodisc, is much less easily affected.

Concerning the relation of the embryo to the blastodisc it might be supposed that, since the two are inevitably connected, a change in the position of one would always be accompanied by a corresponding change in the position of the other. Such a simple statement of the case, however, is subject to important qualifications. In the five instances where the anterior end of the embryo developed at the pole of the egg away from the micropyle the blastodisc had in every case been displaced to the region of the equator (two cases) or below it (three cases), and in each case save one the embryo developed on the same side of the ovum as that originally occupied by the displaced blastodisc. The one case where it developed on the side opposite that to which the blastodisc was moved was not observed during development, but in view of what has happened in other instances to be described presently, it seems probable that the blastodisc in this case eventually moved all the way to the pole. Though only one of these completely reversed embryos developed on the side opposite the one to which the blastodisc was displaced, reference to columns II and IV of table 5 shows that in the case of embryos developing along the sides of centrifuged eggs this curious tendency is very common. Thus in the combined results of early and late centrifuging, column II, 39.54 per cent of the embryos appeared opposite the side of the displaced blastodisc as against 13.95 per cent on the side with the blastodisc, with the remaining 46.51 per cent somewhere in between. Before attempting any explanation of this phenomenon, certain further facts may be indicated as follows:

As suggested, the embryo must, of course, always develop in connection with the blastodisc. Hence in most cases where the former appears on the side opposite that to which the blastodisc was first moved, it must be assumed that some time during development the latter moved back at least as far as the pole. This return to the normal position has in fact been observed in twenty-two out of the seventy-four eggs in which the blastodisc was laterally displaced. These facts, together with the very rare occurrence of embryos developing transversely or across the ends of ova, make it appear probable that there is a tendency for the blastodisc to go either to one pole of the egg or the other. If it is sufficiently far down subsequent to centrifuging, it will go to the pole opposite its original position, and the head end of the embryo will develop at that point; if not, it will usually return to normal.

It is now possible to return to the question of how the changes are brought about in the relations of the blastodisc and embryo to the membrane, as indicated by the shape of the latter and by the position of the micropyle. As previously suggested, apparently either the blastodisc moves over the surface of the yolk, or else the entire egg revolves inside the membrane. The latter has been known to occur in teleost eggs which are spherical, but in the case of eggs shaped as these are, with a membrane which is fairly rigid after short contact with the water, such movement of the egg within it presents some difficulty. This is particularly true of those instances where the relation of the blastodisc and the head of the embryo to the micropyle are completely reversed. Such a case involves the assumption of a very considerable degree of compressibility and elasticity in the substance of the egg. However, there is no good evidence that this does not exist, and considerable reason to think that it does. Indeed, the combination of a certain degree of rigidity and elasticity are just what is needed to account for the special relations of the displaced embryos. Such a situation very readily explains why so few cases of reversal of the anterior-posterior relationship are found, and also why the blastodiscs which have moved less than halfway to the equator tend to move back again as the egg seeks to resume its normal shape within the comparatively rigid membrane. Likewise, the same arguments account for those instances in which the blastodisc, having reached or passed the equator, tends then to go on to the opposite pole. Furthermore, while few of the latter occur, still fewer (only five) have been found in which the blastodisc stopped halfway, and the embryo developed either transversely or across the end of the ovum. Lastly, the one uncentrifuged egg in which the embryo occupied such a position was, as might be expected, almost spherical. These considerations, while not absolutely conclusive, indicate strongly to the writer that movement of the whole egg within its membrane is what probably occurs.

These facts seem clear, but they do not explain the peculiar tendency noted above for the embryo to lie opposite to the side toward which the blastodisc was originally displaced. The only explanation for this which occurs to the writer at present is to assume that the sides of the blastodisc, and perhaps the egg as a whole, differ in specific gravity, and that this difference is definitely related to the future anterior-posterior symmetry of the embryo. If this be true, it brings the evidence from centrifuging into harmony with that from the orientation experiments to the extent that both imply a bilateral symmetry in the egg previous to cleavage which is related to the symmetry of the embryo. Upon the other hand, although the two sets of experiments are in harmony thus far, such data as the orientation work furnishes (see cases of eggs oriented on their sides) seem to show that if gravity is effective at all in placing the embryo, it tends to place it on the higher, and therefore lighter, side of the egg. An analysis of the possibilities of the original positions of ova in the centrifuge, and the possible changes following treatment, however, do not show that the lighter side of the blastodisc (if there be one) would be any more likely to be toward the side from which the blastodisc has moved than toward the other. Yet as indicated, it is on this side that the greater number of embryos in these cases seemed to develop. Hence the meaning of this tendency, if it is confirmed, must await further study.

Summing up the results of the centrifuging experiments, it may be said that on the whole they bear out the conclusion that the egg, shortly subsequent to laying, possesses a definite organization which determines the relation of the embryonic axis to the ovum, and that this organization under normal conditions bears also a definite relation to the egg membrane which can only be altered with considerable difficulty. It seems clear, further, that either the blastodisc, or the end of the egg containing the blastodisc, is of different specific gravity from the other. There is some indication also from this, as well as earlier observations, that the sides of the blastodisc itself, or of the yolk connected with it, likewise differ in weight, and that this may have some direct relationship to the organization referred to.

DISCUSSION

It is not the intention in this paper to consider the relation which the early organization of the egg may have to its later development, except with respect to the determination of the embryonic axis. Nevertheless, it appears appropriate to make a brief reference to this general topic. Numerous investigators have performed experiments of the 'deficiency type' upon fish eggs, including Morgan ('95), Lewis ('12 a and b), and, more recently, Hoadley ('28). Most of this work has been done either for the purpose of studying concrescence or with a view to determining to what extent isolated cleavage cells are able to form the complete embryo. Hoadley, however, has made an especial effort to discover at just what period in development segregation of materials takes place, in such a way, or to such an extent, as to produce real qualitative, as compared with mere quantitative, effects. He concludes that the critical period does not occur until the embryonic-shield stages—a somewhat more advanced condition than that designated by Lewis.

As suggested, it is not felt that these results are particularly germane to the present problem, except insofar as they concern the existence of some organization in the egg after fertilization, perhaps, but previous to cleavage. If such an organization occurs at this stage in the teleost eggs previously worked upon, it apparently involves only the presence of highly modifiable potentialities, potentialities which undoubtedly relate to the position of the embryonic axis as well as other things. The present case is noteworthy in that the existence of an organization relating to the position of the embryo, and present previous to cleavage, seems to have been definitely proved. Evidence has also been adduced which suggests that it is not destroyed, or perhaps even altered, by centrifuging. To what extent it might be affected by operations upon the blastomeres or upon later stages is a problem of considerable interest which remains to be solved.

SUMMARY

The jewel fish, Haemichromis bimaculata, is a tropical aquarium fish which can be made to produce from five to six hundred eggs at periods of from three to four weeks throughout the year. These eggs are of a suitable size and character for embryological study, and are of special interest because of the following facts:

They are ovoid rather than spherical, the blastodisc occurring at one end. Each egg is surrounded by a tough membrane, and is attached to some solid substance by its side. This side, involving both membrane and egg, is flattened slightly, apparently as a result of the pressure of deposition. The embryo then normally develops along the region of the ovum opposite to the side which is flattened, the head appearing at the pole originally occupied by the blastodisc, and the blastopore at the other.

Turning the egg over previous to or during cleavage, so that the flat side is uppermost, does not, in the vast majority of cases, prevent the embryo from developing on the side which it would have occupied had it remained undisturbed. This shows that, whatever influence the relation to the substratum may have initially had, it is no longer effective. There is some evidence, however, that early disturbance other than inversion may tend to upset the normal orientation of the embryo.

Experiments involving the orientation of the egg in such a way that the initially flattened side is vertical and the long axis horizontal, both with and without disturbing the original relation to the substratum, lead to the following conclusions: There seems to be a tendency for the embryo to alter its normal relation to the flat side, so that its sagittal plane approaches the vertical, and so that it lies toward the upper rather than the lower side of the ovum.

In spite of the fact that there may be a slight tendency for the first cleavage plane to be more nearly vertical than horizontal, and under some circumstances at right angles to the flat side of the egg, there is no very consistent relationship between this plane and the axis of the embryo. There may, however, be a slight tendency toward coincidence of these two features.

Centrifuging the eggs at various stages of development previous to gastrulation shows that the original relation of the embryo to its membrane can be changed. The character of these changes, moreover, suggests that they, as well as the variations in orientation previously described, are probably due to a movement of the entire egg within its covering, rather than to a movement of parts of the ovum in relation to each other; i.e., to a change in its organization.

CONCLUSION

The egg of Haemichromis shortly subsequent to laying apparently possesses a definite organization which determines the relation to it of the future embryonic axis. It does not appear that this relation is materially influenced by gravity, and it is probably unaffected by centrifuging. The question as to whether the organization has any relation to the first cleavage plane is not entirely settled, but it appears that there may be sufficient connection to produce a slight tendency for this plane and the sagittal plane of the embryo to coincide. Under these circumstances there are two possible views with respect to the source of this organization. It may be chiefly the result of the pressure of the membrane against one side of the egg at and immediately following deposition. On the other hand, it may be existent in the egg before it is laid. In the latter event it must be assumed that in the vast majority of cases the eggs all pass out of the genital aperture in such a way that their preexistent organization has almost always the same relation to the surface on which they are deposited.

LITERATURE CITED

AGASSIZ, A., AND WHITMAN, C. O. 1884 On the development of some pelagic fish eggs. Preliminary notice. Proc. Amer. Acad. Arts Sci., vol. 20, pp. 23–75.

——— 1885 The development of osseous fishes. I. The pelagic stages of young fishes. Mem. Mus. Comp. Zoöl., vol. 14, no. 1, pt. 1, pp. 1–56.

——— 1889 II. The pre-embryonic stages of development. Part 1. The history of the egg from fertilization to cleavage. Mem. Mus. Comp. Zoöl., vol. 14, no. 1, pt. 2, pp. 1–40.

BATAILLON, E. 1896 Sur les rapports qui existent entre le premier sillon de segmentation et l'axe embryonnaire chez les amphibiens et les téléostéens. C. R. Acad. Sci. Paris, T. 122, pp. 1508–1511.

CLAPP, C. M. 1891 Some points in the development of the toadfish (Batrachus tau). Jour. Morph., vol. 5, pp. 494–501.

DEAN, B. 1895 Fishes living and fossil. Macmillan & Co., New York and London.

HOADLEY, L. 1928 On the localization of developmental potencies in the embryo of Fundulus heteroclitus. Jour. Exp. Zoöl., vol. 52, pp. 7–44.

HOEPFEL, F. 1913 Meine Erfahrungen bei der Zucht von Hemichromis bimaculatus. Wochenschr. Aquar.-Terrar. Kunde, 10. Jahrg., S. 117–118.

KONNERTZ, A. 1913 Entwickelung der Jungfische bei Hemichromis bimaculatus. Wochensch. Aquar.-Terrar. Kunde, 10. Jahrg., S. 941–942.

LEWIS, W. H. 1912 a Experiments on localization in the eggs of a teleost fish (Fundulus heteroclitus). Anat. Rec., vol. 6, pp. 1–6.

——— 1912 b Experiments on localization and regeneration in the embryonic shield and germ ring of a teleost fish (Fundulus heteroclitus). Anat. Rec., vol. 6, pp. 325–333.

MORGAN, T. H. 1893 Experimental studies on teleost eggs. Anat. Anz., Bd. 8, S. 803-814.

——— 1895 The formation of the fish embryo Jour. Morph., vol. 10, pp. 419–472.

PELLEGRIN, J. 1904 Contribution à l'étude anatomique des poissons de la famille des cichlides. Mém. Soc. Zool. France, T. 16, pp. 41–402.

RYDER, J. A. 1882 A contribution to the development of osseous fishes, with special reference to the development of the cod (Gadus morrhua). Rept. U. S. Fish Comm., vol. 10, pp. 455–605.

——— 1886 A preliminary notice of the development of the food fish (Batrachus tau). Bull. U. S. Fish Comm., vol. 6, pp. 4–8.

——— 1890 The origin of sex through cumulative integration and the relation of sexuality to the genesis of species. Proc. Am. Phil. Soc., vol. 28, pp. 109–159.

WILSON, H. V. 1889 The embryology of the sea bass (Serranus atrarius). Bull. U. S. Fish Comm., vol. 9, pp. 209–277.

PLATE 1

EXPLANATION OF FIGURES

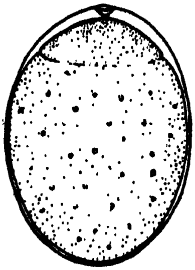
1 Fertilized, but unsegmented egg, shortly before the first cleavage; *a*) as viewed from the side opposite the substratum, i.e., the upper side, *b*) as viewed after turning through 90° with the flattened side toward the reader's right. Note the distinct blastodisc underneath the micropyle. The numerous small circles in the yolk in this and subsequent figures represent the evenly distributed oil droplets.

2 Side view of the animal pole of an egg during fertilization, showing the blastodisc and entrance cone, the latter containing five sperm; *a* marks the point where the lower end of the micropyle is in contact with the entrance cone. A considerable space already exists between the egg and its membrane. The latter often appears thicker than represented here, depending apparently upon the lighting and the angle from which it is viewed.

3 Outlines of an egg in cleavage about five hours after laying; *a*) from the side opposite the substratum, and, *b*) turned 90° to show the flattened region. The blastoderm is in the button-like stage, in which it is raised up and pressed tightly against the egg membrane. The cleavage furrows were too numerous by this time to represent accurately, and so were omitted entirely.

4 An egg about ten hours after laying beginning to gastrulate; *a*) from the side opposite the substratum, and *b*) turned 90°. In the latter note that the blastoderm is farther down on the side to the reader's left, i.e., the region opposite the substratum. This point of greatest growth usually marks the dorsal lip of the blastopore, and hence indicates the axis of the embryo. The membrane in this case appears thicker in places, but this probably is due merely to the angle of illumination across the surface. The suggestion of a slight constriction in the membrane near the animal pole, however, is quite characteristic, though not invariable.

5 An egg about eighteen hours after laying, in which gastrulation is not quite half completed; *a*) viewed from the side opposite the substratum, and, *b*) viewed partly from the animal pole. The embryonic shield is just beginning to be visible in fixed material as a slightly more opaque whitish area. It is represented by shading, and shows best in *b*. The drawings are made from a single egg combining features apparent before and after fixation. Note that all parts of the blastoderm are now equally advanced over the yolk.



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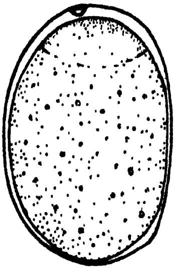

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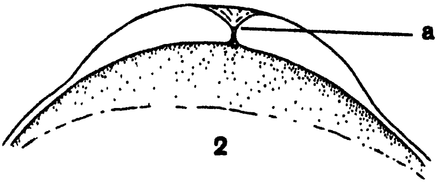

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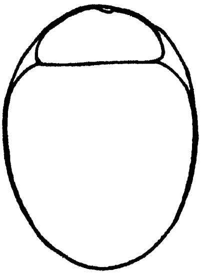

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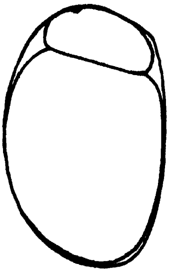

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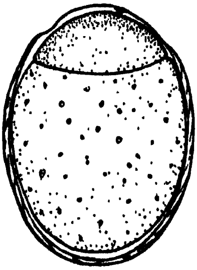

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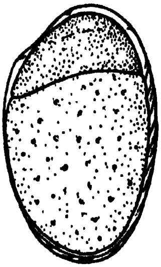

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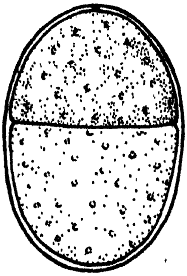

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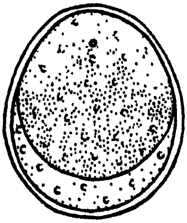


5b

PLATE 2

EXPLANATION OF FIGURES

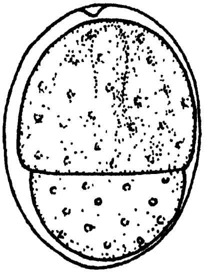
6 An egg about twenty-one hours after laying, showing the blastoderm approximately two-thirds over the yolk; *a*) viewed from the side opposite the substratum, and, *b*) turned 90°. These drawings are again from a single egg both before and after it was fixed. The embryonic anlage has begun to form, but is so transparent as to be scarcely visible in living material except when viewed in profile as in *b*, where it is seen as a thickening on the reader's left. In fixed material it can be observed that this thickening extends to either side of the median line as indicated in both *a* and *b* by shading. A still broader but slighter thickening constitutes the embryonic shield, shown in *a* and *b* by very light shading. In *b* this can be seen extending around the rim of the blastoderm as the germ ring.

7 An egg about twenty-two hours after laying, showing gastrulation nearly completed. In *a* a surface view, and *b*, a profile, the embryonic anlage is clearly outlined, together with the shield extending beyond it. The germ ring is also to be seen surrounding the yolk plug. As usual, these parts are rendered more visible by fixation.

8 An egg viewed from its right side, showing the blastopore closed and the outline of the embryo complete on the reader's left. This is noted as being twenty-two hours after laying, but is obviously slightly further advanced than the egg shown in figure 7.

9 Two eggs viewed from their left sides with their flattened sides in each case to the reader's left. Egg *a* was taken from the crock in late cleavage, and oriented flat side up. Egg *b* was taken from the crock at the same stage as *a* and oriented normally. Note that in each case the embryo has developed on the same side relative to the original flattening. Also note the slight secondary flattening in *a* upon the side where it rested after orientation and upon which the embryo developed.

10 An egg centrifuged for sixty seconds about forty minutes after laying, oriented right side up, and figured about twenty-two hours later. Note the closing blastopore at the wrong end of the membrane directly beneath the micropyle. The egg is in normal position with the side away from the substratum toward the reader. The embryo along the edge of the egg to the reader's left has, therefore, probably been somewhat displaced to one side as well as turned wrong end to. Note also the oil droplets coalesced into globules which now occupy the upper portion of the egg. They are still, however, somewhat toward the side of the embryo, which in this case was also the side occupied by the blastoderm subsequent to centrifuging. This specimen is the single egg recorded in table 5, batch III, columns IV and V.



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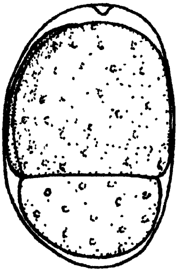

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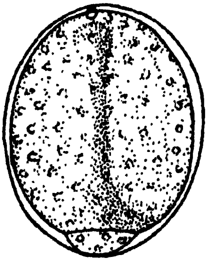

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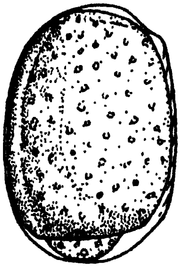

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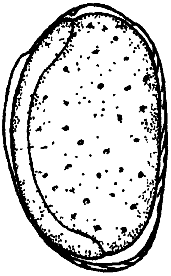

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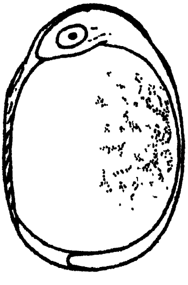

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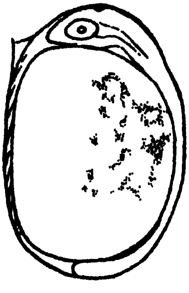

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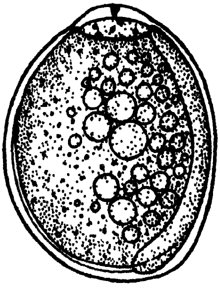

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